STUDIES ON THE EXPRESSION OF THE GENES FOR THE THIRD AND FOURTH COMPONENTS OF THE COMPLEMENT SYSTEM

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STELLINGEN

1. De hypothese van Ooi et al., dat processing van pro-C5 tot C5 het verschil in de relatieve hoeveelheden van C5-antigeen en C5-activiteit kan verklaren is eenvoudig te testen.

Ooi,Y.M., Harris,D.E., Edelson,P.J. and Colten,H.R. (1980). Post-translational control of complement (C5) production by resident and stimulated mouse macrophages. J. Immunol. 124: 2077-2081.

2. Het is voorstelbaar dat oorspronkelijk slechts twee eiwitten betrokken waren bij de activering van het complement systeem. Dupliceringen van genen, gevolgd door mutaties kunnen hebben geleid tot het onstaan van C2 en factor B enerzijds en C3, C4 en C5 anderzijds.

Fernandez,H.N. and Hugli,T.E. (1977). Chemical evidence for common genetic ancestry of complement components C3 and C5. J. Biol. Chem. 252: 1826-1828.

Lachmann,P.J. and Hobart,M.J. (1979). The genetics of the complement system. in: "Human Genetics: possibilities and realities", Ciba Foundation Symposium 66, new series, (Porter,P.J. and Hobart,M.J. eds.), Excerpta Medica, Amsterdam: 231-250.

3. Het feit, dat C3 een groot aantal verschillende functies heeft, rechtvaardigt inderdaad de pogingen om de gehele structuur van C3 op te helderen. Aangezien complementair DNA voor C3 beschikbaar is geworden, is het thans uitvoerbaar om de gehele aminozuurvolgorde van C3 te bepalen.

Müller-Eberhard,H.J. and Schreiber,R.D. (1980). Molecular biology and chemistry of the alternative pathway of complement. Adv. Immunol. 29: 1-53.

4. Het is uitgesloten dat het mechanisme van de antigene variatie van Trypanosoma brucei gelijk is aan het mechanisme dat genen voor specifieke immunoglobulinen in B-cellen tot expressie brengt, aangezien T. brucei een monocellulair organisme is.

Hoeijmakers,J.H.J., Frasch,A.C.C., Bernards,A., Borst,P. and Cross,G.A.M. (1980). Novel expression-linked copies of the genes for variant surface antigens in trypanosomes. Nature 284: 78-80.

Max,E.E., Seidman,J.G. and Leder,P. (1979). Sequences of five potential recombination sites encoded close to an immunoglobulin κ constant region gene. Proc. Nat. Acad. Sci. USA 76: 3450-3454.

Sakano,H., Hüppi,K., Heinrich,G. and Tonegawa,S. (1979). Sequences at the somatic recombination sites of immunoglobulin light-chain genes. Nature 280: 288-294.

5. Zelfs als met behulp van recombinant DNA technieken een vaccin tegen malaria geproduceerd kan worden, zal de bestrijding van deze ziekte niet eenvoudg zijn, vanwege de vele sociale complicaties in de gebieden waar malaria endemisch is.

6. Het glycosoom, een organel dat gespecialiseerd is in de glycolyse, biedt de Trypanosomatiden een interessante mogelijkheid voor een glycolyse die ATP levert in afwezigheid van zuurstof.

Opperdoes,F. and Borst,P. (1977). Localization of nine glycolytic enzymes in a microbody-like organelle in Trypanosoma brucei: the glycosome. FEBS letter 80: 360-364.

7. Het is onwaarschijnlijk, dat bij de vermeerdering van kernpolyeder virussen in insectecellen, de synthese van het polyedereiwit verloopt via een precursoreiwit.

Carstens,E.B., Tjia,S.T. and Doerfler,W. (1979). Infection of Spodoptera frugiperda cells with Autographa californica nuclear polyhedrosis virus. 1. Synthesis of intracellular proteins after virus infection. Virology 99: 386-398.

Van der Beek,C.P., Saayer-Riep,J.D. and Vlak.J.M. (1980). On the origin of the polyhedral protein of Autographa californica nuclear polyhedrosis virus. Isolation, characterization and translation of viral messenger RNA. Virology 100: 326-333.

Dobos,P. and Cochran,M.A. (1980). Protein synthesis in cells infected by Autographa californica nuclear polyhedrosis virus (Ac-NPV): the effect of cytosin arabinoside. Virology 103: 446-464.

8. Er is geen experimenteel bewijs beschikbaar voor het bestaan van een fragment in het provirale DNA van het muizen borst kanker virus, dat de moleculaire cloning van het complete virale DNA in E. coli onmogelijk zou maken. Indien dit "giftige" fragment bestaat, dan lijkt het niet essentieel te zijn voor de expressie van het virale DNA.

Majors,J.E. and Varmus,H.E. (1981). Nucleotide sequences at host-proviral junctions for mouse mammary tumor virus. Nature 289: 253-262.

Buetti,E. and Diggelmann,H. (1981). Cloned MMTV DNA is biologically active in transfected mouse cells and its expression is stimulated by glucocorticoid hormones. Cell 23: 335-345.

9. Het verband tussen de lengte van de werkweek en de hoeveelheid geslaagde proeven is verre van linear.

Proefschrift van K.G.Odink.
Studies on the expression of the genes for the third and fourth components of the complement system.
Wageningen, 25 juni 1981.

CONTENTS

PROMOTIE IN DE AULA VAN DE LANDBOUWHOGESCHOOL
GENERAAL FOULKESWEG 1a

RECEPTIE NA AFLOOP VAN DE PROMOTIE
IN HET KLEIN-AUDITORIUM VAN HET AULAGEBOUW

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.1 Purpose of the investigations

The work reported in this thesis forms part of a research project, defined o study some problems related to the immune response by means of recently eveloped techniques of molecular biology. The major aim of this project is o investigate the expression of the genes for the third and fourth components f the complement system.

The complement system defends man and other vertebrates against infections. set of at least sixteen serum proteins is involved in the activation of the ystem, which can be induced by antigen-antibody complexes and a variety of ther substances. The activation of the system comprises a cascade series of roteolytic cleavages and protein binding interactions, leading to the rapid learance of infectious agents from the inflamed site. The regulation of the xpression of the genes involved in the complement system is obscure. A struc- ural analysis and study of the transcription of these genes can help to eluci- ate these problems. In addition these studies can give an understanding of enetically determined deficiencies in these components.

The development of recombinant DNA techniques recently has made it possible o adress these questions. Messenger RNA molecules (mRNA) can be used as template or reverse transcription into complementary DNA (cDNA), which can be inserted nto the DNA of bacterial plasmids. Bacteria are transformed with this recombi- ant DNA and in this way pure complementary DNA can be isolated in large amounts rom cultures of transformed bacteria. Due to the complementarity of this DNA o the corresponding RNA it is refered to as cDNA-probe. DNA probes can be used n hybridization studies for the identification of messenger RNA and the corres- onding gene. For instance, RNA and DNA samples can be size fractionated by gel lectrophoresis, followed by transfer and binding onto a solid support (eg. nitro- ellulose filters). The position of the specific RNA or DNA bands can subsequent- y be visualized by hybridization to a radioactive DNA probe followed by auto- adiography. In a similar way DNA probes can be used for the quantification of pecific RNA and DNA and for the determination of the gene copy number. The solation of gene fragments is monitored using DNA probes and finally the nucleo- ide sequence of the DNA probe can be determined.

For several reasons the genes for the complement components C3 and C4, are of particular interest. a) C3 is an essential component in the complement activation pathways and probably the most important component of the whole system (see 1.3.4 and the figure on page 4). b) the C4-gene(but not the C3-gene) is located in the major histocompatibility complex (MHC, ref. 1). The MHC of the mouse represents the best characterized cluster of mammalian genes and is of great interest to immunologists, since it discriminates self from non-self and provides information about the genetics of biological phenomena. Access to the C4-gene would provide an entrance for studies of gene arrangement in the MHC. c) an allogenic variant for mouse C4 exists, the Slp or sex-limited protein, the expression of which exhibits a strong dependence on testosterone (2, 3). d) feasibility reasons : C3 and C4 are the most abundant complement components in serum (see table 1) and have comparable high molecular weights; therefore their mRNAs can be identified and many steps in the molecular cloning can be performed for both in parallel (see 1.2 and chapter 3). We chose the murine complement system because the mouse is an animal for which many well defined inbred strains exist. This might allow later a comparison of differences in the DNA structure with differences in the genotype and phenotype.

The work which is reported here (chapters 2,3 and 4) describes the attempt which were made in order to generate DNA probes complementary to C3- and C4-mRN and the use of the isolated DNA probe for C3 for the comparison of C3-mRNA levels in cells.

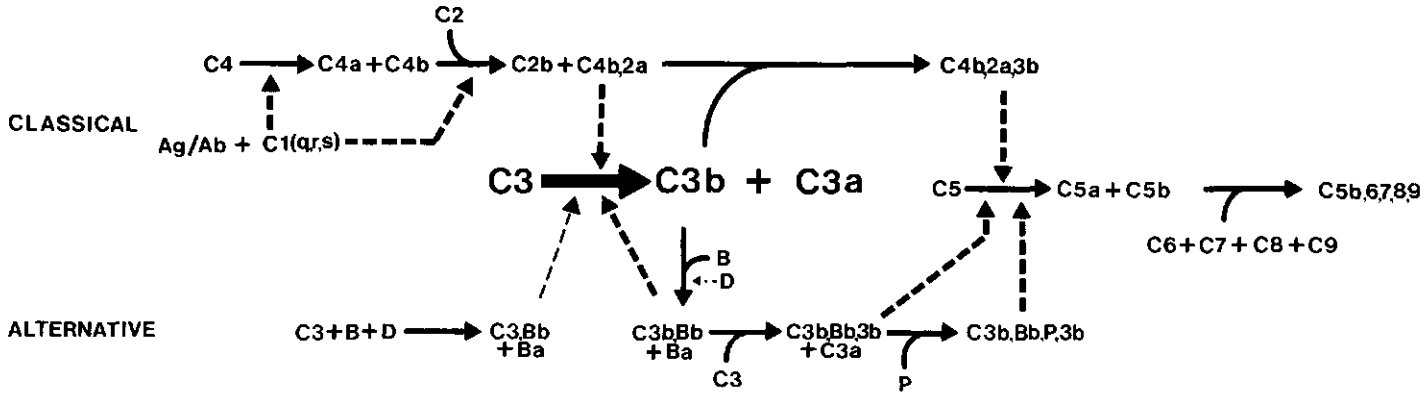
1.2. Strategy

The number of specific mRNAs from eucaryotic cells for which cDNA has been cloned in bacteria is increasing rapidly and the technology of molecular cloning has been greatly improved (4, 5, 6). This does not mean that for any given mRNA it is easy to identify clones which contain its corresponding cDNA. In the first place a specific assay must be available for the protein encoded by the mRNA for the identification of this mRNA. This assay must be easy and sensitive, especially when the abundancy of the specific mRNA is low. Only in few cases it is possible to purify extensively a given mRNA when this is only present in low amounts. Therefore the molecular cloning of a specific

DNA usually starts with a mixture of cDNAs and the cloning procedure provides for an absolute purification. However if the concentration of cDNA of interest is low, a high number of individual bacterial clones has to be screened for the presence of this cDNA.

Two methods are mainly used for the identification of cDNA contained in bacterial clones, both based on in vitro translation of mRNA which hybridizes specifically to the cDNA. In the first method, the hybrid promoted translation 7), specific mRNA is translated in vitro after it has been isolated from a complex mixture by virtue of its capacity to hybridize to the cDNA, in a way similar to affinity chromatography. In the second method, the hybrid arrested translation (8), a mixture of mRNA is translated in vitro, but a specific mRNA is blocked by hybridization to cDNA, thereby causing the disappearance of the protein corresponding to the blocked mRNA. Many proteins are synthesized in the form of a precursor which subsequently is cleaved and modified. Because the in vitro translation systems usually do not process the newly synthesized proteins, it is important that an assay system is available that detects not only the mature protein but also its possible precursors.

For the precursors of C3 and C4, pro-C3 and pro-C4, no assays are available for their functional activity, but they can be identified by immunoprecipitations with specific antisera for C3 and C4. Hybrid promoted and arrested translations followed by immunoprecipitations are technically difficult and time consuming and for that reason do not allow an easy screening of a large number of cDNA clones. Since the mRNA concentrations for C3 and C4 in their major producer cells, the hepatocytes, are estimated to be only 0.1-0.5% of the total mRNA (9, 10, 11), it was necessary to enrich for the C3- and C4-mRNA prior to the molecular cloning procedure. For both C3 and C4 it has been reported that they are synthesized as single chain precursors of 180-200 kilodaltons and therefore their mRNAs were expected to fall into the size class of at least 5000 bases (28S). Because the average chain length mRNA in liver is 2000 bases (18S), it was anticipated that the mRNAs for C3 and C4, should be separable from the bulk of the mRNA by size selection. The overall strategy for the cloning of DNA complementary to C3- and C4-mRNAs sequences was as follows : a) generation and identification of an antiserum against mouse C4 (chapter 2); anti mouse C3 serum is commercially available. b) size fractionation of mRNA and identification of C3- and C4-mRNA by in vitro translation and immunoprecipitation (chapter 3). c) generation of



THE COMPLEMENT SYSTEM

Schematic representation of the classical and alternative pathways of complement activation and the assembly of the membrane attack complex. Enzymatic reactions and/or formation of (enzyme)complexes are indicated by solid arrows (⟶). Enzyme activities exerted in reactions are indicated by broken arrows (-----›). Ag/Ab stands for antigen-antibody complexes, for further nomenclature see 1.3.2 and for a detailed description 1.3.3 .

ouble stranded cDNA with the size selected RNA as template; insertion of it nto a bacterial plasmid and transformation of E.coli cells with the recombi- ant plasmid (chapter 3) d) screening of isolated clones for the presence of NA inserts which are complementary to mRNA of a size corresponding with 3- and C4-mRNA (chapter 3) and characterization of the putative C3 and C4 lones by in vitro translation of mRNA selected by specific hybridization (7) ollowed by immunoprecipitation (chapter 3)

.3 The complement system

.3.1 The role and action of the complement system

The complement system is a set of at least sixteen proteins in blood lasma and body fluids of vertebrates which plays an important role in the efence mechanism against infections. The system accomplishes the clearance f material of both foreign origin, such as bacteria, viruses, etc., and of he organism itself, eg. aged cells and cell debris, by the lysis of cells by lisruption of their membranes and/or phagocytosis.

A schematic representation of the system is depicted on page 4 (for a letailed description see 1.3.3). The system is divided into two pathways, for istorical reasons called classical and alternative pathway. They represent wo cascade series of proteolytic cleavages, both leading to the same important esults, namely the cleavage on a large scale of C3 into C3a and C3b and the leavage of C5 into C5a and C5b (for nomenclature see 1.3.2). The fragment C3b, hen bound in a large number to targets, facilitates the phagocytosis of these argets by white blood cells. The fragment C5b mediates the assembly of a rotein complex (C5b, 6, 7, 8, 9 outer right in the figure) which can cause lysis of target cells by disruption of their membranes. Several small polypep- ides, C4a, C3a and C5a, generated by the cleavage of some of the components f the complement system, are known to cause tissue inflammation (see anaphyla- oxins, 1.3.5). Normally this inflammation favors a fast removal of the infec- ious agent, followed by healing. However prolonged inflammation can cause erious tissue damage.

The classical activation pathway is induced by antigen-antibody complexes. he mechanism of initiation of the alternative activation pathway is ill

TABLE 1. THE COMPONENTS OF THE COMPLEMENT SYSTEM AND THE FRAGMENTS WHICH ARISE DURING THEIR ACTIVATION

Component	Fragment	Molecular Weight 1)	Polypeptide Chains 1,2)	Concentration in Serum (µg/ml)
C1q		410'000	A(6) 24'000 B(6) 23'000 C(6) 22'000	150
C1r		83'000		50
C1s		83'000		50
C2		110'000		15
	C2a	70'000		
	C2b	30'000		
C3		180'000	α 120'000 β 70'000	1200
	C3a	9'000		
	C3b	170'000		
C4		200'000	α 98'000 β 70'000 γ 30'000	400
	C4a	6'000		
	C4b	170'000		
C5		180'000	α 105'000 β 75'000	70
	C5a	10'000		
	C5b	170'000		
C6		95'000		60
C7		110'000		55
C8		163'000	α 83'000 β 70'000 γ 10'000	55
C9		70'000		60
B		93'000		200
D		24'000		1
P		224'000	(4)[3] 55'000	20
I		88'000	50'000 38'000	34
H		150'000		500

1)values for the components in human serum (14,15,16).Differences between independant reports and various species usually do not exceed 10% of these values. 2)determined by denaturing gel electrophoresis after reduction.The number of chains is indicated between brackets.The chain(s) of the fragments and components with only one chain are not indicated.3)probably identical.

nderstood. This pathway is stimultated by binding of C3b molecules on target
iaterial (see 1.3.3).

.3.2 Nomenclature of the complement system

The components of the complement system are designated here according
:o two accepted conventions (12, 13). Two activation pathways are defined, the
:lassical and alternative pathways, both leading to the assembly of the same
iembrane attack complex. The classical activation pathway comprises the proteins
:1, C2, C3 and C4; the alternative activation pathway is effected by the pro-
:eins C3, B, D and P and in addition two proteins with a regulatory function,
iamely H and I. The proteins that are involved in the membrane attack are
iumerically designated as C5, ..., C9. Component C1 is a monomolecular complex
›f the proteins C1q, C1r and C1s. The letters a, b, ..., f after the component
lesignation, eg. C3a and C3b, refer to the fragments which result from proteo-
ytic cleavages during activation of the complement system. Greek letters,
:g. C3α and C3β, refer to individual polypeptide chains which arise after dena-
:uration and reduction of native components. (Enzyme) complexes of proteins are
indicated by the capital letter C, followed by an enumeration of the numbers
ind symbols of the proteins, with an intervening comma, eg. C4b,2a and C3b,Bb.

All components with their approximative molecular weights, polypeptide
:hains and major fragments are listed in table 1.

1.3.3 Activation of the complement system

The activation of both the classical and alternative pathways has been
:xtensively reviewed (14, 15, 16, 17). Here only the basic mechanism of activa-
:ion, which is schematically drawn on page 4, will be summarized.

The classical pathway of complement activation is initiated by the presence
›f antigen-antibody complexes of IgM and IgG type. The immune complex is
:ecognized by C1, which binds at the second constant domain of immunoglobulins.
:1 is a complex of three components, C1q, C1r and C1s, held together by Ca^{++} -
ions. The binding of C1 to immunoglobulins probably causes a conformational
:hange in C1r, through which it becomes activated and in turn can activate C1s
›y a proteolytic cleavage. Activated C1s is a protease and catalyses the

hydrolysis of a serine ester bond in C4, thereby forming C4a and C4b. The C4b molecule posseses an active binding site for membranes, cell surfaces and particles. Because the active binding site has a very short half-life, the C4b molecule can only bind close to the site of its generation. C2 can associate with bound C4b, after which it can be cleaved into C2a and C2b, again by the activated Cls in the Cl complex. The C2a in association with the C4b molecule can form C4b,2a which is a C3-convertase. One C3-convertase molecule (C4b,2a) can split several hundred molecules of C3 into the fragments C3a and C3b. Part of the C3b molecules bind to C4b,2a under formation of C4b,2a,3b, which is a C5-convertase, but the majority can adhere elsewhere to the surface of the same cell or particle, thus enhancing phagocytosis (see 1.3.4).

Both the C3-and C5-convertases (C4b,2a and C4b,2a,3b) have a limited life span due to the fast loss of C2a which is rapidly inactivated in solution after its dissociation from the complex. The last proteolytic step in the pathway is the cleavage of C5, catalyzed by C5-convertase (C4b,2a,3b) which results in the formation of C5a and C5b. Finally C5b initiates an assembly process of itself with C6, C7, C8 and C9 (for review see 18). The complex C5b,6,7,8,9 which is created by this self assembly process is responsible for the membrane attack leading to cell lysis. It is noteworthy that five different soluble proteins become peripheral or possibly integral membrane constituents after this process of self assembly.

The inflammation causing effect of C4a, C3a and C5a will be described below (1.3.5).

The alternative activation pathway can be triggered in vivo and in vitro by a variety of substances, including antigen binding fragments in immune complexes, bacteria, protozoan parasites, animal cells, yeast cell walls and collagen structures of vascular basement membranes. It is thought that continuously a soluble C3-convertase is formed by the association of C3 with factor B in presence of factor D. The C3b molecules which are formed at a slow rate by this initial soluble C3-convertase can bind to many kind of particulate material. If the bound C3b does not rapidly become inactivated (see below), factor B can associate with it. Subsequently factor D cleaves the bound factor B into Ba and Bb and C3b,Bb is formed which is a bound C3-convertase. Then the process becomes amplified because C3b,Bb provides for a control of the generation of C3b by a positive feedback. Deposition of more C3b close to C3b,Bb leads to the

ormation of C3b,Bb,3b which is a C5-convertase and cleaves C5 into C5a and 5b. The C5b molecules initiate in the same way as in the classical pathway the ormation of C5b,6,7,8,9 the membrane attack complex.

It is postulated that the alternative pathway mainly is controlled by the ate of inactivation of C3b. Two proteins, factor I and H, are involved in the nactivation of C3b (not indicated in the figure). Factor I can cleave a peptide ond in the αchain of C3b which then becomes inactive. Factor H can bind to C3b hat impairs the binding of factor B, accelerates the dissociation of Bb from he C3b,Bb complex and thereby facilitates the inactivation of C3b by factor I. he susceptibility of C3b to factors I and H is influenced in an unknown way y features of the substances to which C3b is bound. There are "activating" ubstances which slow down the inactivation of C3b what results in the stimula- ion of the pathway.

In addition to the control just described, both the classical and alterna- ive pathways are further regulated, in order to prevent large scale destruction n the organism. This is achieved by the cascade nature of the activation which llows rapid activation but makes at the same time precise regulation possible y inhibition of crucial steps in the pathway. The extent of activation is imited by inhibition of the convertase activities and the disassembly of the abile convertases. Furthermore, due to the short half lives of their activated inding sites, C3b and C4b can only bind close to the site of their generation. his prevents non-target material to become involved in the action of the comple- ient system. The activation of C1 is controlled by C1 esterase inhibitor.

.3.4 The role of complement component C3

Genetically determined deficiencies are known for nearly all complement :omponents in man and animals (for review see 19, 20, 21), but only the (almost) :omplete deficiency in C3 in man clearly has been related to a high susceptibi- ity to infections (19). This is easily understood as C3 is the only essential :omponent in both pathways (see the figure on page 4) and C3 is a constituent)f most of the convertases.

The major role of the complement system probably resides in the process)f phagocytosis and in the generation of small polypeptides C3a, C4a and C5a /hich initiate tissue inflammation (see anaphylatoxins, 1.3.4). The phagocytosis

of foreign- and self-material (bacteria, viruses, aged cells, etc.) is promoted by C3b. During the amplification step in the complement activation, C3→C3a + C particles can become coated with C3b. This causes the immune adherence of these particles to C3-receptors present on a variety of cells (primate red blood cell non-primate platelets, lymphocytes, polymorphonuclear leucocytes, monocytes and macrophages), followed by ingestion and consecutive enzymatic hydrolysis by enz enzymes from the granula of the white blood cells.

The membrane attack complex mainly has been studied by the immune lysis of red blood cells in vitro, the socalled immune hemolysis. It is not clear to what extend the process of membrane attack is essential for the organism.

1.3.5 Anaphylatoxins

The polypeptides C3a, C4a and C5a, which are called anaphylatoxins, cause tissue inflammation (14, 17, 22). They are generated by proteolytic cleavage of the chains of the complement proteins. Among their effects are smooth muscle contraction, increase of vascular permeability, which causes a high influx of plasma proteins and white blood cells in the inflammed tissue. Further they cause the release of histamine and serotonin from mast cells upon binding to receptors on these cells. Prolonged inflammation can lead to serious damage of the tissue, eg. necrosis and the formation an an abscess.

C5a can mediate yet another important event namely chemotaxis (23), which is the directional migration of cells towards a "chemotactic" agent. C5a attracts polymorphonuclear leucocytes and monocytes to the site of inflammation It enhances the activation of a proesterase of these cells, which is thought to facilitate the passage of the white blood cells through the blood vessel wall.

1.3.6. Biosynthesis of the complement components

1.3.6.1 Producer cells and precursors

The synthesis of complement components can be detected by 1) measurement of the activity of a component in a functional assay, eg. hemolytical assay; 2) identification of newly synthesized proteins by immunological means, eg. immunoprecipitation after metabolic labeling of proteins. 3) detection of mRNA

for complement components, eg. by in vitro translation followed by immunoprecipitation.

It has been shown that gut-associated cells, hepatocytes and epithelial cells of the genito-urinary and intestinal tracts, and cells of the reticulo-endothelial system, monocytes and macrophages, are major producers of complement components (for review see 24, 25, 26). It is not known whether a single cell is able to synthesize more than one complement component. Several types of cells in tissue culture produce complement components. Among these are cells of fibroblastoid (27, 28, 29) and hepatic origin (28, 30) as well as somatic hybrids between these two cell types (31).

In their mature form several of the complement components are glycosylated (32, 33) and consist of multiple chains (see table 1), some of which are linked by disulfide bonds. Probably these separate polypeptide chains arise from a common precursor by posttranslational processing. For some of the complement components, notably C3, C4 and C5 single chain polypeptides have been detected by immunoprecipitation, with a size which would be expected for a single chain precursor (see table 2). For mouse C4 pulse chase experiments (32, 34) and for human C3 (35) and mouse C4 (34) tryptic peptide analysis have shown that these single chain molecules indeed represent precursors for the mature proteins. In addition, for both C3 and C4 only high molecular weight mRNAs were detected in mouse liver, which coded for C3 and C4 polypeptides of precursor length (36 chapter 3).

1.3.6.2 Regulation of the biosynthesis of the complement components

The regulation of complement biosynthesis is subject to both genetic and non-genetic controls. The former has been extensively reviewed (19) and will not be discussed here. Non-genetic controls, effectuated by micro-environmental facters such as hormones and inflammatory agents, form interesting model systems to study the molecular mechanism of regulation of gene expression. However such a study is complicated because a) in in vivo experiments it is difficult to distinguish direct action from indirect action, which may involve a multitude of substances; b) in vitro studies using cell lines are less complex but not always of biological relevance and c) the assays mentioned above (1.3.6.1) are also used to measure the amounts of complement components. But

TABLE 2. SINGLE CHAIN POLYPEPTIDES OF THE COMPLEMENT COMPONENTS C3, C4 AND C5.

	Species	Site of Detection	Molecular Weight 1)	References
Pro-C3	Mouse	liver	175'000*	36
	Mouse	liver	200'000*	37
	Mouse	macrophage	175'000	38
	Rabbit	liver	200'000*	11
	Guinea pig	liver	170'000	10
	Human	plasma	197'000	35, 39
Pro-C4	Mouse	liver	190'000*	36
	Mouse	macrophage	185'000	34, 40
	Mouse	macrophage	200'000	32
	Mouse	liver	200'000	9
	Guinea pig	plasma	200'000	41
	Guinea pig	plasma	200'000	42
	Human	plasma	200'000	
Pro-C5	Mouse	liver	170'000*	37
	Mouse	liver	200'000	37
	Mouse	macrophage	210'000	43

1) values obtained by gel electrophoresis under denaturing and reducing conditions. The values marked by an asterisk were obtained by in vitro translation of mRNA.

the level of complement activity of a complement protein, the amount of a complement protein as measured as antigen, and the level of its mRNA are not necessarily similar. Therefore different methods to determine the amounts of complement components can lead to different results and interpretations (Chapter 4, 44, 45).

The level of several complement components in serum, measured mainly by their hemolytic activity, is influenced by hormones (for review see 24, 26, 46). For instance in male mice the levels of some components are up to ten fold higher than in female mice (46). In this respect it is noteworthy that, in contrast to

:4, the level of the inactive variant of C4, the sex limited protein or Slp (2), s strongly dependent on the presence of testosterone (3). The production of :3-activity by a well differentiated rat hepatoma cell line is reported (30) to ncrease up to nine fold in the presence of hydrocortisone. In contrast the same glucocorticoid hormone reduced the synthesis of C2-activity in primary macro-)hages (28). The synthetic glucocorticoid dexamethasone reduces the production of :3-protein in several cell lines (chapter 4).

The serum levels of several complement components are increased, as measured)y activity, following generalized infection and large tissue traumas, the so-:alled acute phase (14, 47). Infections and inflammations can be stimulated in laboratory animals by intra-peritoneal injections of several infectious and irri-tating agents such as bacteria and starch (48, 49). These treatments attract many nacrophages to the peritoneum. For guinea pigs it has been shown that the propor-tion of macrophages which synthesize hemolytically active C2 and C4 remains un-altered upon injection of starch (44). For reasons mentioned above it is diffi-cult to intrepret the results of the measurements of the amounts of newly synthe-sized complement components in such experiments (see also the discussion in chapter 4). The rate of synthesis of active C2 and C4 by guinea pig macrophages increased (44) but for active C5 a decrease was measured (46) upon starch induc-tion. In contrast in these experiments the synthesis of C2, C4 and C5 antigen did not change significantly. At present it is not clear whether these discre-pancies reflect effects on the processing of precursor proteins into mature and active components and the matter can be considered to be completely open.

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Synthesis of the mouse complement component C4 (Ss-protein) by peritoneal macrophages: kinetics of secretion and glycosylation of the subunits*

Ss antigen was partially purified from mouse EDTA plasma, and a highly specific antiserum was prepared. Peritoneal exudate cells from Ss-high mouse strains were labeled in primary tissue culture with [^{35}S]methionine. Ss protein was found to be synthesized by macrophages and to be secreted into the culture medium as shown by indirect immunoprecipitations performed with this antiserum. The unreduced precipitated Ss molecule coelectrophoresed with human C4 at an apparent mol. wt. of 200000. Upon reduction, Ss protein dissociated into three subunits α, β and γ of mol. wts. 98000, 70000 and 32000. This pattern was almost identical to the one obtained for human C4. The Ss α subunit was converted by human $\overline{C1s}$ into an α subunit of approximately 85 Kdalton. This conversion is a characteristic property of C4. A single-chain intracellular precursor for the Ss protein (pre-Ss: 200 Kdalton) was also immunoprecipitated. In pulse-chase experiments, it disappeared from the cytoplasmic extract within less than 10 h in a reciprocal manner to the appearance of extracellular mature Ss protein. No significant amounts of high-mol. wt. extracellular precursors were detected. A group of four single-chain molecules of mol. wt. between 160000 and 220000 was immunoprecipitated from the tissue culture supernatant with anti-Ss antiserum. These molecules could not be chased into mature Ss protein. The α, α′ and β subunits of Ss and the α chain of C3 incorporated [^{3}H]mannose and [^{3}H]glucosamine, the Ss γ and C3 β chains did not.

1 Introduction

Genes in the S region of the major histocompatibility complex (MHC) of the mouse control the serum levels of the Ss protein which is now known to be the fourth component (C4) of the complement system [1–4]. A variant of Ss, the Slp protein (sex-limited protein), is present only in the serum of males of certain mouse strains which carry a dominant autosomal gene for Slp [5]. The expression of the Slp protein is strongly dependent on testosterone [6]. Slp does not mediate C4 hemolytic function [4], and it is not known whether it carries other functions. Both the Ss and Slp polypeptides are glycosylated. During their secretion from producer cells they appear to be transiently associated with the outer cell membrane. In this phase, they may be involved in immunological mechanisms other than the classical complement functions [7]. For this reason, S-region products have recently received much attention in fundamental immunological research. Also, they are interesting because they can be used as markers for the fine-structure analysis of the MHC on the genome level. Finally, the expression of the Slp protein is a particularly clear-cut example for control by steroid hormones in the mouse.

Liver parenchymal cells and peritoneal macrophages have been demonstrated to be the principal producer cells of the complement components C3 and C4 in mouse and guinea pig [8, 9]. In particular for the mouse, in only one report [10] have the molecular structures of the products synthesized by macrophages been studied in detail. Single-chain precursor molecules have been reported for both C3 and C4 in guinea pig and man [11–14]. Again, in the case of the mouse high-mol. wt. single chain, molecules which immunologically cross-react with the mature Ss protein have been demonstrated intracellularly [10], but until now the precursor-product relationship has not been carefully demonstrated by pulse-chase experiments and comparative peptide analysis.

The present report describes a new procedure for the partial purification of Ss antigen and for the generation of a rabbit antiserum of high titer and specificity. Using this antiserum for immunoprecipitations of metabolically labeled products from peritoneal exudate cells in primary tissue cultures, it was confirmed that macrophages indeed synthesize significant amounts of Ss protein and secrete it into the culture medium. The molecular structure of the immunoprecipitated material was studied in detail and compared to human C4 and mouse C3. C3 was chosen as a specificity control throughout this work because it resembles C4 in size and chemical properties. However, the C3 gene has been mapped outside of the mouse MHC [15], and no steroid hormone control over levels of C3 has been reported.

[I 2501]

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Abbreviations: CIG: Cold insoluble globulin **DTT:** Dithiothreitol **PBS:** Phosphate-buffered saline **PEG:** Polyethylene glycol **PMSF:** Phenyl methyl sulfonyl fluoride **PEP:** PBS buffer supplemented with EDTA and PMSF **SDS:** Sodium dodecyl sulfate **SDS-PAGE:** SDS-polyacrylamide gel electrophoresis **TCA:** Trichloroacetic acid **VBS:** Veronal-buffered saline **VBS^{++}:** VBS supplemented with 1 mM Mg^{++} and 0.15 mM Ca^{++} **MHC:** Major histocompatibility complex

Complement components are abbreviated according to the recommendations of the Committee on Complement Nomenclature of the World Health Organization (*Bull. WHO* 1968. *39:* 935). C3 and C4 designate the third and fourth components, $\overline{C1s}$ the activated form of C1s. The subunits are labeled α, β and γ according to the convention, whereas we used α′, α″, α‴ to designate fragments of the α chain, for which no official symbols have yet been proposed.

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An intracellular single-chain high-mol. wt. precursor for Ss was detected, and its processing into mature extracellular products was studied by pulse-chase experiments. Finally, using metabolic labeling with various radioactive sugars, the glycosylation of the individual subunits of Ss protein was investigated.

2 Materials and methods

2.1 Partial purification of Ss antigen

Blood was collected individually from three-month-old male DBA/2J mice into ice-cold 20 mM EDTA (final concentration). Plasma samples were pooled and phenyl methyl sulfonyl fluoride (PMSF) was added to a final concentration of 0.1 mg/ml. Plasma proteins were precipitated with polyethylene glycol (PEG) 6000, and the 5–15% fraction was recovered essentially as described by Bolotin et al. [16]. The precipitate was dissolved in 10 mM sodium phosphate buffer, pH 7.1, containing 0.15 M NaCl, 1% glycerol, 10 mM EDTA and 0.1 mg/ml PMSF. The solution was dialyzed overnight against 10 mM sodium acetate buffer, pH 5.3, with 5 mM EDTA and 0.1 mg/ml PMSF. The precipitate was redissolved in the same sodium phosphate buffer as above. It was dialyzed against 50 mM sodium phosphate buffer, pH 8.2, containing 5 mM EDTA and 0.1 mg/ml PMSF and applied to QAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with the same buffer. The column was developed with a gradient of sodium chloride from 0 to 0.5 M in the same buffer. In all purification steps, fractions positive for Ss antigen were identified by double diffusion against a reference anti-Ss antiserum (kind gifts from Drs. T. Meo and D. Shreffler). Positive fractions were pooled, precipitated with ammonium sulfate at 50% saturation, resuspended in phosphate-buffered saline (PBS) containing 10 mM EDTA, dialyzed against the same buffer and used for immunization.

2.2 Purification of human C4, human $\overline{C1s}$ and mouse cold insoluble globulin (CIG)

Human C4 was isolated from freshly frozen citrated plasma by sequential ion exchange chromatography on SP-Sephadex and QAE-Sephadex (R. M. Chapuis, manuscript in preparation). A rabbit antiserum was prepared against the purified C4 protein and the IgG fraction prepared by standard procedures. Reference anti-human C4 antiserum was purchased from Nordic Labs., Tilburg, NL. Human $\overline{C1s}$ was purified as described elsewhere [17], omitting the precautions to prevent activation of C1. Mouse CIG was purified using published procedures [18], and an antiserum against CIG was obtained from rabbits.

2.3 Preparation of anti-mouse Ss antiserum

Rabbits were immunized by subcutaneous injections at multiple sites with approximately 600 µg of partially purified material in complete Freund's adjuvant. After three weeks, the animals were boosted 2–3 × at weekly intervals with approximately 200 µg of the same material in incomplete Freund's adjuvant. For the absorption of the serum, the 15% PEG precipitable fractions from EDTA plasma of C3H and CBA/CaJax females (both H-2^k, Ss low) were combined in equal portions and coupled covalently to Sepharose 4B using the cyanogen bromide procedure [19]. The IgG fraction of the absorbed serum was then used for large-scale preparative immunodiffusions. The precipitin lines which corresponded to those obtained with reference serum were cut out from the agar, washed exhaustively with PEP buffer (PBS containing 10 mM EDTA and 0.1 mg/ml PMSF) plus 0.5 M NaCl and subsequently with PEP buffer alone. They were homogenized in a 1 : 1 mixture of the same buffer with complete Freund's adjuvant and used for immunization of a second series of rabbits. The amount of precipitated protein which each animal received per injection was estimated to be 25–50 µg. The final serum was again absorbed with Ss-low plasma and the IgG fraction prepared as above: it detected the Ss antigen in EDTA plasma of two Ss-high mouse strains (C57BL/6 males and DBA/2J males). No precipitin lines were observed with plasma from two Ss-low strains (CBA/Ca females and C3H females). In immunodiffusion tests, the antiserum gave rise to a line of complete identity with the reference anti-Ss antiserum (Fig. 1).

2.4 Primary tissue cultures of peritoneal cells and metabolic labeling

Adult males from Ss-high strains (C57BL/6, DBA/2J) were killed by cervical dislocation and injected twice intraperitoneally with 5 ml of ice-cold Eagle's medium containing 10% fetal bovine serum, L-glutamine at 0.22 mg/ml and lacking methionine, if metabolic labeling was to be performed with radioactive methionine. If radioactive sugars were to be used, complete medium was taken. Peritoneal cells were transferred into an ice-cold centrifuge tube, sedimented, resuspended in 0.4 ml of prewarmed medium and immediately plated out into plastic tissue culture petri dishes of 6 cm diameter. Each dish received the cells from four mice, corresponding approximately to 4×10^6 macrophages. The cells were incubated at 37 °C with 100–500 µCi of labeled precursors per plate. The spec. act. were approximately 100 Ci/mmol (= 3.7 TBq) for [^{35}S] methionine and 10, respectively 19 Ci/mmol, for mannose

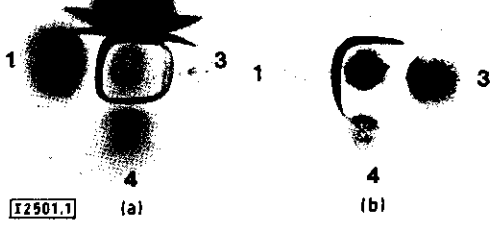


Figure 1. Characterization of anti-Ss antisera by double diffusion.
(a) Demonstration of the effect of absorption. Center well: EDTA plasma from C57BL/6 males; (1) reference anti-Ss antiserum (Meo); (2) unabsorbed anti-Ss antiserum (Lausanne); (3) anti-Ss antiserum, same as in (2), but absorbed with pooled plasma proteins from C3H and CBA/Ca females; (4) same as (3), IgG fraction.
(b) Reaction of absorbed anti-Ss antiserum with plasma from various mouse strains. Center well: absorbed anti-Ss antiserum (Lausanne). Periphery: EDTA plasma from (1) C57BL/6 males; (2) DBA/2J males; (3) C3H females; (4) CBA/Ca females. Note: very low amounts of Ss are also detected in plasma of Ss-low strains.

ıd glucosamine. When it was attempted to distinguish ;tween synthesis by adherent cells and nonadherent cells, tachment was first allowed to proceed for 1 to 2 h, then the lls were washed twice, and only then fresh medium and label ere added. Cytoplasmic extracts were prepared by incubatg the adherent cells on the petri dish for 15 min on ice with 5 ml of PEP buffer containing 0.5% Nonidet-P40 (NP40). uclei and membrane debris were removed by centrifugation r 5 min at 5000 rpm and the supernatant used as cytoplasmic :tract.

5 Immunoprecipitations

issue culture supernatants were concentrated approximately refold by dialysis against PEP buffer containing 15% PEG. he samples were first cleared from material which sticks nspecifically to *Staphylococcus aureus* by exhaustive reincubation with *Staph. A* (formaldehyde-fixed bacteria, rain Cowan I), [20]. Sequential precipitations were then cared out with anti-CIG, anti-C3 and anti-Ss antisera. After ich incubation, the complexes were precipitated with *aph. A*. Rabbit anti-rat C3 was purchased from Nordic abs. and found to cross-react strongly with mouse C3. Comexes were washed 4 × with 1 ml each of PEP buffer containg 0.5% NP40 (twice), PEP plus 0.05% NP40 (once) and nally PEP buffer alone. The immune complexes were eluted rice with 50 µl of SDS (sodium dodecyl sulfate) sample buffr [21] without reducing agents by boiling for 3 min and centifugation.

6 Analysis of immunoprecipitates by SDS polyacrylamide gel electrophoresis (PAGE)

he SDS-PAGE slab gel system was used as described by ämmli [21] with only half the amounts of bisacrylamide in the acking gels. Samples were reduced with 25 mM dithiothreitol)TT) and alkylated with a 2.2-fold molar excess of doacetamide in SDS sample buffer.

Results

.1 Synthesis of the Ss protein by peritoneal cells

1.1 Adherent *vs.* nonadherent cells

/hen the total population of peritoneal exudate cells was laced into culture and labeled immediately with [^{35}S]methionine, Ss protein was clearly synthesized and secreted into the tissue culture medium (Fig. 2b). When adherent cells were allowed to attach first for 2 h and nonadherent cells were washed away, immunoprecipitable material with a similar electrophoretic pattern of bands was synthesized (data not shown). Thus, in agreement with others [10], we concluded that adherent peritoneal cells (macrophages) were capable immediately after seeding into tissue culture of synthesizing Ss protein and did not need the continuous presence of other cells or factors secreted by other cells. However, the absolute amounts of immunoprecipitable, radioactively labeled protein was severalfold lower when cells were allowed to attach first. We have not observed synthesis of Ss protein from macrophages when labeling was performed after five days of culture. At this time, the attached cells still continued to synthesize and secrete detectable amounts of C3. We conclude that attached macrophages within a few days in tissue culture strongly reduce the level of newly synthesized and secreted Ss. A similar observation has been made by Roos et al. [10]. In addition, we were unable to detect synthesis of Ss protein from starch-stimulated macrophages, although these continued to synthesize significant amounts of C3. For these reasons, all data shown below were obtained using unstimulated total peritoneal cell populations, labeled immediately after establishment of the tissue culture.

3.1.2 Subunit structure of the extracellular Ss molecule

In most experiments, the Ss material which was immunoprecipitated from tissue culture supernatants gave rise after reduction and alkylation to three predominant bands upon SDS-PAGE (Fig. 2b). These have apparent mol. wts. of 98000, 70000 and 32000. The number of three subunits and their apparent mol. wts. correspond well to the values reported by Roos et al. [10]. We therefore identified these bands as Ss (α), Ss (β) and Ss (γ). This identification was supported by several additional findings: these subunits compared well to those of human C4 (see below), the same bands in identical relative amounts were precipitated with anti-human C4 antiserum (Fig. 2d), but not with anti-CIG antiserum nor with anti-C3 antiserum (Fig. 2e, f). In addition to these major bands, we regularly observed minor bands (see Sect. 3.1.3) which have not been described in the report by Roos et al. [10]. The autoradiograph shown in Fig. 2b was scanned in a recording spectrophotometer. A semiquantitative evaluation was possible because it was exposed as a fluorogra-

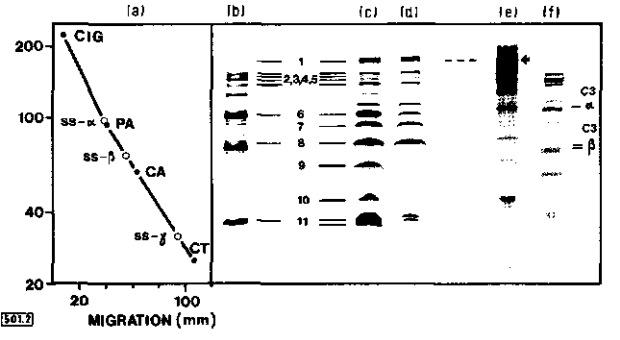


Figure 2. Subunit structure of reduced immunoprecipitated Ss molecules. Electrophoresis in 15% SDS-polyacrylamide gels.
(a) Mobility markers: phosphorylase A (PA, 94 Kdalton), catalase (CA, 60 Kdalton), and chymotrypsinogen (CT, 25.5 Kdalton). (b, c) Two independent experiments with anti-Ss antiserum. The numbers of bands are explained in Table I. (d) Precipitation with anti-human C4 antiserum from same macrophage culture supernatant as (c). (e, f) Sequential precipitates with anti-CIG (e) and anti-C3 (f) from same source extract as (c). Precipitations (e) and (f) were performed prior to precipitation (c).
Track 2b is a fluorography exposed on preflashed Kodak RP Royal X-Omat film [22]. The intensity of the preflash was adjusted such that the response curve of the film was linear between 0.15 and 2.2 bsorbance units at 540 nm. The peak intensities of bands α, β and γ fell into the linear range so that a quantitative evaluation was possible from scan of this autoradiography.

Table 1. Molecular weights of immunoprecipitated components

Band	Identification	Mol. wt. ($\times 10^{-3}$)
1	CIG[a]	230
2	Ss-related[b]	175
3	molecules	170
4	(?)	160
5		150
6	Ss α	98
7	Ss α′	85
8	Ss β	70
9	Ss α″ (?)	55
10	Ss α‴ (?)	40
11	Ss γ	32

a) Band 1 is identified as CIG by specific immunoprecipitation with anti-CIG (Fig. 2e) and by comigration with purified CIG marker protein.
b) Bands 2–5 are the high-mol. wt. single-chain components which are described in the text; they are believed to be antigenically related to Ss.
c) Bands 9 and 10 are tentatively designated breakdown fragments of Ss-α, because they appeared only when α′ was seen and because human C4 α, but not β and γ subunits, are specifically cleaved into similar-sized fragments [4].

phy on preflashed film ([22], see legend to Fig. 2). The α, β and γ bands accounted for 21, 21 and 19.8%, respectively, of the total immunoprecipitated radioactivity. The γ component was seen in several but not all experiments as a doublet.

3.1.3 Minor immunoprecipitated components

Some of the minor components which were specifically precipitated with our anti-Ss antiserum were found reproducibly, others in varying amounts in different experiments. We numbered them from the top to the bottom of the electrophoretic pattern in Fig. 2c (Table 1). The group of bands 2, 3, 4 and 5 (discussed in Sect. 3.3.3) comprised together 18.2% of the total immunoprecipitated radioactivity.

3.2 Comparison of mouse Ss and human C4 proteins

3.2.1 Size of the unreduced and reduced molecules

Additional proof that the immunoprecipitated Ss protein is mouse C4 was provided by comparison of its molecular structure with that of radioiodinated [23], purified human C4. It has previously been shown by others that the number of subunits is the same and that their sizes are very similar for mouse and human C4, and that these two cross-react immunologically [3, 10]. Macrophage tissue culture supernatants were fractionated on an LKB Ultrogel AcA 22 column in the presence of 1.15 M NaCl prior to immunoprecipitation in order to avoid trapping of unreduced immunoprecipitated material on top of the gel. The Ss-containing fractions were pooled and concentrated by dialysis against PEP buffer containing 15% PEG. Sequential immunoprecipitations with anti-CIG, anti-C3 and anti-Ss antisera were performed, and the precipitates were analyzed under reducing (Fig. 3a) and nonreducing (Fig. 3b) conditions by SDS-PAGE on 15% and 7.5% gels, respectively. The precipitation with anti-CIG was performed in order to generate more specific immunoprecipitation patterns, because pilot experiments had shown that our anti-Ss antiserum contained even after absorption significant amounts of contaminating anti-CIG antibodies. The mol. wt. of the unreduced Ss protein coincided exactly with that of unreduced human C4 (Fig. 3b, tracks 1 and 4) and was clearly different from unreduced CIG (Fig. 3b, track 2 marked by arrow) and unreduced C3 (Fig. 3b, track 3, arrow). In addition, the material precipitated with anti-Ss contained a component of an apparent mol. wt. comparable to C3 (Fig. 3b, track 4). This is probably contaminating C3 which was not completely removed in the preceding immunoprecipitation step. In the reduced pattern (Fig. 3a, track 3) one clearly detects contamination by C3 subunits. The unreduced C4 molecule migrated approximately as the reduced intracellular Ss precursor molecule (Fig. 3b, tracks 4 and 5). Upon reduction and alkylation, human C4 dissociated into three major bands: α (94 Kdalton), β (72 Kdalton) and γ (30 Kdalton) (Fig. 3a, track 5), which migrated with very similar mobilities as Ss (α), Ss (β) and Ss (γ) (Fig. 3a, tracks 3 and 4) and appeared to be quite different from the α and β subunits of C3 (Fig. 3a, track 2).

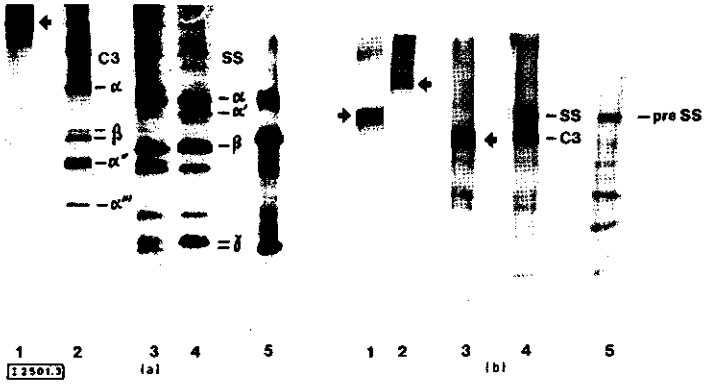


Figure 3. Comparison of unreduced (b) and reduced (a) Ss and pre-Ss with human C4. Macrophage culture supernatants fractionated on Ultrogel AcA 22 column. Ss-positive fractions pooled and sequentially immunoprecipitated. Precipitates analyzed by SDS-PAGE in (a) 15% gels (reduced samples) and (b) 7.5% gels (unreduced samples).
(a) (1–3) Precipitates with anti-CIG, anti-C3 and anti-Ss, respectively; (4) precipitate with anti-Ss from unfractionated macrophage culture supernatants; (5) human C4, purified and radioiodinated with ^{125}I.
(b) (1) human C4; (2–4) precipitates with anti-CIG, anti-C3 and anti-Ss, respectively; (5) reduced precipitate with anti-Ss from a cytoplasmic extract of adherent macrophages in tissue culture, showing pre-Ss.

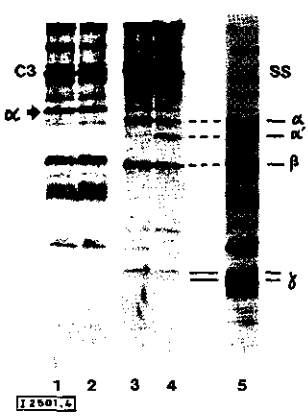
2.2 Susceptibility of Ss (α) to human $\overline{C1s}$

ne of the characteristic properties of the mouse Ss protein, y which it can be distinguished even from the closely related p protein, is that its α subunit can be specifically cleaved by man $\overline{C1s}$ [4]. In order to further confirm the identity of the s material precipitated by our antiserum, we have treated ooled Ss-positive fractions from macrophage tissue culture pernatants purified on Ultrogel AcA 22 columns (as above) ith $\overline{C1s}$. We were able to demonstrate the generation of Ss ') from Ss (α) by the action of $\overline{C1s}$ (Fig. 4, tracks 3 and 4). he result confirmed our belief that the precipitated material as mouse C4 because the action of $\overline{C1s}$ is highly specific for 4 α. Indeed, the treatment in our experiment did not affect ie subunits of C3.

.3 Ss precursor molecules

.3.1 Kinetics of appearance of Ss in tissue culture supernatants

n an attempt to identify precursors and to demonstrate pre-ursor-product relationships we have performed pulse-chase xperiments. Unstimulated total peritoneal exudate cells were abeled immediately after establishment of tissue cultures for h with [^{35}S]methionine. The tissue culture supernatants at ie end of this pulse labeling were recovered and stored. The ultures were washed and incubated in complete medium for arious lengths of time from 1–18 h. After harvesting of the supernatants, the adherent cells were washed, and cytoplasmic extracts were prepared. Aliquots of tissue culture supernatants and cytoplasmic extracts were removed for precipitation with trichloroacetic acid (TCA). Then, sequential immunoprecipitations were performed on all samples, and aliquots were taken again to determine the proportion of immunoprecipitable material. The kinetics of appearance of total newly synthesized radiolabeled protein in the tissue culture supernatant is given in Fig. 5a. The total exported acid-precipitable radioactive protein reached a constant concentration after 4 h of chase. The increase of immunoprecipitable material with anti-Ss antiserum in the tissue culture supernatant is shown in Fig. 5b, expressed as percentage of the total extracellular acid-precipitable material. At most 5% of the newly synthesized and secreted proteins are reacting with anti-Ss antiserum. As shown in Fig. 2b, at least 60% of the reactive material are Ss molecules. This plateau level is in very close agreement with the value given by Roos et al. [10].



igure 4. Action of human $\overline{C1s}$ on mouse Ss and C3.
DS-PAGE of reduced samples in a 15% polyacrylamide gel. The Jltrogel AcA 22 column was equilibrated with VBS^{++} (containing alcium and magnesium), and the Ss-positive fractions were concentrated by dialysis against 15% PEG in VBS^{++} followed by brief dialysis against VBS^{++} without PEG. To 1 ml samples of the concentrate vere added either buffer (control) or 20 μg $\overline{C1s}$. Samples were incubated 16 h at 0°C. EDTA was added to 10 mM and PMSF to 0.1 mg/ nl, and sequential immunoprecipitations, reduction, alkylation and electrophoresis were performed as above.
1, 2) Precipitates with anti-C3; (3, 4) precipitates with anti-Ss, samples *treated with buffer and* $\overline{C1s}$, *respectively; (5) reference precipi*ate with anti-Ss from an unfractionated macrophage culture supernatant.

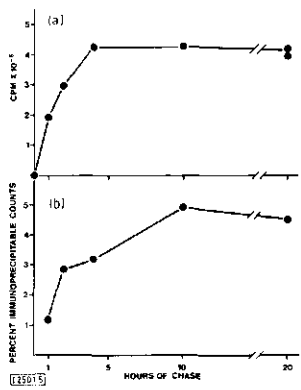


Figure 5. Kinetics of appearance of Ss antigen in macrophage tissue culture supernatants.
Primary cultures were labeled for 2 h with [^{35}S]methionine, and chases were performed as described. Equal aliquots from the supernatants after various times of chase were precipitated with TCA or immunoprecipitated with anti-Ss antiserum (sequentially after anti-C1G and anti-C3).
(a) Total TCA-precipitable counts from tissue culture supernatants of adherent cells pooled from 4 mice (approximately 4×10^6 adherent cells) labeled with 0.1 mCi of [^{35}S]methionine; (b) total immunoprecipitable counts from the same source extracts as in (a) expressed as percentage of total TCA-precipitable protein.

3.3.2 Intracellular precursors

PAGE analysis of the immunoprecipitated material from the pulse-chase experiments is shown in Fig. 6a for tissue culture supernatants and in Fig. 6b for the corresponding cytoplasmic extracts. A specifically precipitated high-mol. wt. single-chain protein (labeled pre-Ss in Fig. 6b) was present in the cytoplasm immediately after the end of the pulse (Fig. 6b, tracks 2 and 3). Upon chases of increasing lengths, it disappeared from the cytoplasm (Fig. 6b, tracks 4, 5 and 6) while at the same time mature Ss protein accumulated in the extracellular compartment (Fig. 6a, tracks 3 to 6). We therefore concluded that this was the major intracellular precursor for the Ss molecule and named it pre-Ss. This interpretation still needs to be con-

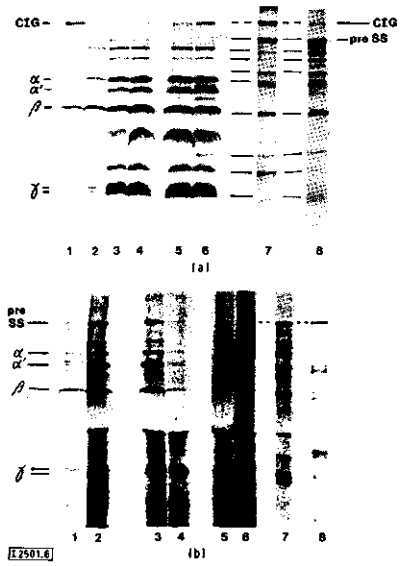


Figure 6. Pulse-chase experiments.
SDS-PAGE of reduced immunoprecipitates in 15% polyacrylamide gels.
(a) Precipitates from tissue culture supernatants, tracks 1 to 6 (Exp. 1), chase intervals of 0, 1, 2, 4, 10 and 16 h; tracks 7 and 8 (Exp. 2), chase intervals of 0 and 19 h, respectively.
(b) Precipitates from cytoplasmic extracts, tracks 1 to 6 (Exp. 1), chase intervals of 0, 1, 2, 4, 10 and 16 h; track 7 (Exp. 2), chase interval of 8 h; track 8 (Exp. 3), 20 h continuous labeling.
Precipitates 1 to 6 in panels (a) and (b) were sequential precipitates after pre-exhaustion with anti-CIG and anti-C3; however, pre-exhaustion with anti-CIG was incomplete. The position of CIG was identified by reference to a cold, purified CIG-marker which was included in the same gel.

firmed by comparative peptide analysis. We cannot yet assign this molecule a reliable mol. wt., because in our experiments it migrates as approximately 200 Kdaltons in nonreducing, and as approximately 150 Kdaltons component in reducing gels. It corresponds in size to band 2 (Table 1) of the extracellular components. Mature α, α', β and γ chains were also detectable in low amounts in cytoplasmic extracts in some experiments (Fig. 6b, tracks 1 to 3). Most of this material disappeared from the cytoplasm in the same time as the major precursor pre-Ss (Fig. 6b, tracks 4 to 6). However, from a different type of experiment we concluded that no large pools of mature products accumulated in the cytoplasm. In this type of experiment, the labeling was performed continuously for 20 h in order to visualize the steady-state levels of intracellular Ss-related molecules. The result (Fig. 6b, track 8) shows that a significant pool of pre-Ss but no significant pools of mature products were present. This result demonstrates that the presence of mature α, α', β and γ chains in cytoplasmic extracts (Fig. 6b, tracks 1 to 4) is overaccentuated by pulse-chase experiments which draw the attention to newly appearing material. The equilibrium concentrations of mature components in the cytoplasm, which are represented by continuous labeling experiments, are negligible with respect to the precursor pre-Ss. From a third, independent experiment we have obtained suggestive evidence that the processing from pre-Ss to mature products requires living cells. In this particular experiment, the cells died during the labeling pulse due to the use of a batch of fetal calf serum different from that used in the other experiments, which were performed later with specially selected batches of serum. Upon microscopic inspection, the cells offered a very different morphological aspect from normal macrophages at this stage, and upon washing at the end of the pulse, unusually many of them detached and gave rise to debris in the wash liquid. Immunoprecipitations were carried out as in the previous experiments from cytoplasmic extracts and tissue culture supernatants taken immediately after the pulse and after increasing intervals of chase. Fig. 6b, track 7, shows that intracellular precursor pre-Ss was synthesized, but that after 8 h of chase it was not completely delivered from the cytoplasm, as opposed to the corresponding situation in normal cells (Fig. 6b, track 5) where export after this time was essentially complete. Fig. 6a, tracks 7 and 8, show the corresponding analysis of the extracellular material after 0 h and 19 h of chase. Large amounts of pre-Ss and only very minor amounts of mature α, β and γ chains were detected even after 19 h of chase. In the normal situation (Fig. 6a, track 6), no extracellular pre-Ss was seen, but large amounts of mature chains. We interprete this as leakage of intracellular pre-Ss from dead cells into the tissue culture supernatant, where it was not properly processed. We suggest that processing into mature products requires living cells and is not likely to be achieved by stable pools of extracellular processing enzymes. There are still minor differences detectable between the patterns of immunoprecipitated proteins from the supernatants of dead cells after 0 and 19 h of chase (Fig. 6a, tracks 7 and 8). We have not investigated these in detail, but one likely explanation is that, whereas proteolytic processing ceases rapidly upon cell death, some other form of processing (glycosylation) might still continue in addition to an increased leakage of cytoplasmic material from dead cells into the extracellular compartment during 19 h of chase.

In the experiments with healthy cells, we observed that already after zero time and 1 h of chase (Fig. 6a, tracks 1 and 2), the majority of Ss-reactive extracellular material was present as mature α, α', β and γ chains. The processing must therefore have occurred either very rapidly in the extracellular space or during the secretion from the cells. Because we failed in the experiment with dying cells to observe processing by extracellular proteases, we favor the view that processing takes place during the release from the cells.

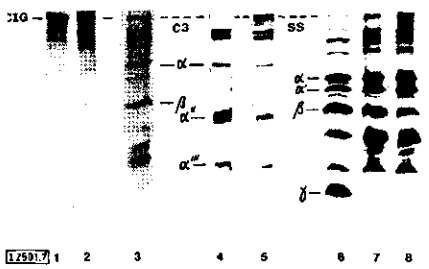
3.3.3 Extracellular Ss-related single-chain components

High-mol. wt., single-chain polypeptides (Table 1, Bands 2, 3, 4 and 5) have reproducibly been specifically precipitated with our anti-Ss antiserum. All of these together comprised 18% of the total immunoprecipitated radioactive protein in Fig. 2b. In pulse-chase experiments (Fig. 6a, tracks 1 to 6), they do not initially comprise the majority of radioactive material and do not subsequently decrease in a reciprocal way to the appearance of mature triple-chain Ss molecules. Therefore, it is unlikely that they are major extracellular precursors for mature Ss. These components can also be precipitated in very similar relative proportions with anti-human C4 antiserum (Fig. 2d). A similar pattern yet distinct and specific was also observed in several experiments for C3 (Fig. 2f). These poly-

eptides are probably dead-end products of aberrant processing pathways or, less likely, simple contaminants of our immunoprecipitations.

4 Glycosylation of the subunits

nstimulated peritoneal exudate cells were labeled in tissue ulture with [^{3}H]mannose and [^{3}H]glucosamine. Sequential mmunoprecipitations from culture supernatants and subsequent electrophoretic analysis were then performed as above. s shown in Fig. 7, tracks 7 and 8, Ss (α), Ss (α′), Ss (β) and me of the high-mol. wt. single-chain Ss-related molecules ere labeled with both mannose and glucosamine. The Ss (γ) ubunit did not incorporate either one of these sugars. CIG rried both sugars (Fig. 7, tracks 1 and 2), and so did C3α, hereas we failed to detect significant amounts of these sugars C3β (Fig. 7, tracks 4 and 5). Two single-chain high-mol. t. bands which were specifically precipitated with anti-C3 rum, had also incorporated these sugars (Fig. 7, tracks 4 nd 5).



igure 7. Incorporation of mannose and glucosamine into Ss and C3. DS-PAGE of reduced samples in 15% polyacrylamide gels. 1, 2) Precipitates with anti-CIG labeled with mannose and lucosamine, respectively; (3–5) precipitates with anti-C3 labeled vith [^{35}S]methionine (3), [^{3}H]mannose (4), [^{3}H]glucosamine (5); 6–8) precipitates with anti-Ss labeled with [^{35}S]methionine (6), ^{3}H]mannose (7), [^{3}H]glucosamine (8). Fluoroautoradiography with reflashing and exposure on Kodak RP Royal X-Omat film was performed according to Laskey and Mills [22].

5 Discussion

One of the purposes of the present work was to produce an anti-Ss antiserum as a reagent for further studies on the expression of the Ss and Slp genes. We have described the production of the anti-Ss antiserum and the efforts to establish its specificity. A few remarks now follow concerning the production of the serum. The precipitation of Ss at the isoelectric point in low salt concentration was included as a rapid initial purification step. Others [24, 25] have successfully used the same technique independently, and different partial purification procedures have been briefly described [5, 26]. It does not seem to be of critical importance which procedure is used to partially purify the antigen prior to immunization. Anti-Ss antisera have been obtained even by immunization with total mouse globulins [1]. In view of the low concentration of Ss in EDTA plasma (0.2% of total protein) it must be concluded that Ss is a highly immunogenic molecule. The specificity of an anti-Ss antiserum can be greatly enhanced by absorption of the crude antiserum with plasma proteins from Ss-low mice. It was of substantial advantage to pool for this purpose plasma proteins from several different Ss-low mouse strains. The efficiency of the absorption was increased by coupling the adsorbing agents covalently to Sepharose 4B. The regeneration of the columns was optimized by using as desorbing agent 0.1 M glycine at pH 2.5. The following observations showed that the antiserum precipitated Ss in a highly specific manner: the number and size of the subunits and the size of the unreduced molecule matched the reported values [10]; the size of the precipitated molecule and its subunit composition was very similar to human C4 and different from mouse C3; the molecule was synthesized by the expected cell type and was susceptible to specific proteolytic attack by human $\overline{C1s}$; the precipitable molecules were present in plasma from two Ss-high mouse strains in much higher concentrations than in the plasma from two Ss-low strains.

Several new facts emerged about the synthesis and secretion of the Ss molecule and its glycosylation:

(a) A single-chain, high-mol. wt. precursor for Ss (pre-Ss) was found intracellularly, which in pulse-chase experiments showed the appropriate behavior for a major precursor.

(b) A group of four extracellular high-mol. wt. proteins was detected of size between 150 and 220 Kdaltons, which are probably antigenically related to the Ss molecule. They are single-chain components which could not be chased into mature Ss protein. At least two of these were found to be glycosylated. A similar group of several high-mol. wt. single-chain polypeptides was also precipitated with anti-C3 antiserum from macrophage culture supernatants. However, these proteins were distinct in their exact mol. wts. from those precipitated with anti-Ss antiserum.

(c) Under steady-state labeling conditions, no large pools of mature intracellular Ss molecules were found to accumulate, and no large amounts of major extracellular precursors were detected. Thus, the processing from the single-chain precursor into the triple-chain mature product is likely to occur in a timed correlation with the secretion of the molecules from the producer cells.

(d) The α, α′ and β chains of Ss and C3α incorporated mannose and glucosamine, whereas we could not detect these sugars incorporated into Ss γ and C3β chains.

(e) Peritoneal macrophages in primary tissue culture ceased to secrete significant amounts of Ss molecules into the culture medium within 48 h, whereas they continued to secrete detectable amounts of newly synthesized C3. These observations confirm the original findings of Roos et al. [10]. The synthesis of C3 and C4 might be subject to different control mechanisms, because the genes for these two proteins are located in distant regions of chromosome 17 of the mouse [15, 27].

Our data suggest the existence of a major intracellular precursor (pre-Ss) which under steady-state conditions was present in excess concentrations over mature Ss chains. This precursor had been previously decribed by others [10]. The new finding is that in chase experiments, it disappeared in good kinetic correlation with the appearance of extracellular mature products. The details of the processing scheme have still to be elaborated. A mechanism of cell-associated proteolytic cleavage is compatible with our data, but a very rapid extracellular cleavage could not be completely excluded. This would have to take place so fast that already after 1 h of chase, no extracellular precursor would be left.

The extracellular high-mol. wt. single-chain polypeptides, which were precipitated with anti-Ss antiserum, could not be chased into mature products and, therefore, are not functional precursors. They could be present due to aberrant processing at a low level. Because a similar but distinct group of molecules was specifically precipitated with anti-C3 antiserum, we favor the interpretation that these molecules are antigenically related to C4 and C3, respectively. The trivial explanation that they are due to contaminant antibodies in the sera has not been ruled out. So-called single-chain "precursors" have also been observed at a level of less than 5% of the mature product in mouse EDTA plasma [10] and in human plasma [13, 14]. In these cases, a precursor-product relationship has not been demonstrated, and therefore those molecules should not be called "precursors" yet.

It appears unlikely that one of the radioactive components immunoprecipitated from macrophage culture supernatants is the C4b-binding protein described by Ferreira et al. [28]. The Ss protein, which was used for the first cycle of immunization, was partially purified from EDTA plasma. For the second round of immunizations, precipitin material was used which was again generated from EDTA plasma and should not contain the C4b-binding protein. Thus, our anti-Ss antiserum is not likely to contain anti-C4b-binding protein antibodies. It is also unlikely that the C4b-binding protein should be coprecipitated with Ss protein from the macrophage tissue culture supernatant, because the majority of the material is present in our experiments as C4, whereas the C4b-binding protein has been shown to bind specifically to C4b [28]. All the immunoprecipitations were carried out in presence of EDTA and protease inhibitors in order to prevent the conversion from C4 to C4b. Finally, it is not known whether C4b-binding protein is synthesized by peritoneal macrophages in primary cultures in sufficient amounts to be visible in autoradiographs of newly synthesized, secreted proteins.

We can also exclude that one of these high-mol. wt. proteins is related to the Slp protein, because the macrophages used in this experiment were taken from C57BL/6 mice which are Slp-negative.

Mannose and glucosamine are abundant sugars in glycoproteins, and mannose is found as a core sugar almost universally [29]. The fact that we did not find these sugars in the Ss (γ) chain makes it likely that this chain is not glycosylated. Glycosylation of the γ chain of human C4 has not been firmly established. Marginal sugar contents have been reported for it [30, 31] which in both reports could not be quantitated. Human C3 has been reported to contain between 1 and 3% of carbohydrates [32, 33], but the glycosylation of the subunits has not been studied.

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Mouse Complement Components C3 and C4

CHARACTERIZATION OF THEIR MESSENGER RNA AND MOLECULAR CLONING OF COMPLEMENTARY DNA FOR C3*

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Mouse liver mRNA species which direct the cell-free synthesis of pro-C3 and pro-C4 polypeptides with an apparent molecular weight of 175,000 and 190,000, respectively, were shown to sediment faster than 28 S. By electrophoresis in a denaturing gel the mRNA for C3 was determined to contain approximately 7,500 ribonucleotides. cDNA was synthesized from size-fractionated mouse liver mRNA and cloned in the plasmid pBR 322. Among the cDNA clones recovered three were identified as being complementary to portions of the mRNA for C3.

The third and fourth components of the complement system (C3 and C4) are serum glycoproteins, which in mammalian organisms participate in defense reactions against infections (1). C3 is an essential element in both the classical and the alternative pathways of complement utilization. While partial C3 deficiencies in humans have been reported, complete C3 deficiencies are rarely observed, due to fatal childhood infections (2). In contrast, complete homozygous C4 deficiencies without associated serious disease have been detected in humans (3) and guinea pigs (4). Humans have two separate loci for two distinct hemolytically active C4 proteins (5, 6). In mice only one gene is known for hemolytically active C4. Mice, however, possess a variant of the C4 protein (Slp or sex-limited protein), which is probably the product of a duplicated gene (7, 8) but which has no C4-hemolytical function (9).

Human and mouse C4 genes are located inside the major histocompatibility complex (HLA, H-2), whereas the human C3 gene is not linked to the HLA complex (10) and the mouse C3 gene is located distant from the H-2 complex on the same chromosome (11).

C3 is synthesized as an intracellular precursor (pro-C3) of an approximate molecular weight of 170,000 which is processed by proteolytic cleavage into two mature chains α, 120,000 and β, 70,000 (12, 13). Similarly, C4 is synthesized as a single chain precursor (pro-C4) with an approximate molecular weight of 200,000. It is cleaved into a disulfide-bonded triple chain with subunits α (98,000), β (70,000), and γ (32,000) with minor species variations (14–16).

The major site of synthesis of C3 (and probably C4) is the liver (17). Secondary sites are the peritoneal macrophages (15, 16) and a few other cell types (18, 19). The biosynthesis of C3, C4, and other complement proteins can be regulated by hormones (18), and in particular, the mouse Slp protein is strongly dependent on testosterone (7).

Our goal is to study the structure and the arrangement of the genes for C3 and C4, the control of their expression, and their alterations in inherited disorders. As tools for these studies we have attempted to clone cDNA sequences of C3 and C4. We report here: 1) the characterization of the sizes and cell-free translation properties of the mRNAs for C3 and C4, and 2) the construction and identification of recombinant plasmids containing sequences complementary to mRNA for C3.

EXPERIMENTAL PROCEDURES

Animals—Adult DBA/2J male mice were used for all experiments except that mRNA from C57/BL6 adult males was used for the synthesis of cDNA. Both mouse strains are kept as continuous stock in the animal facility of our institute.

Materials—Oligo(dT)-cellulose type II was purchased from the Collaborative Research Inc. Anti-C3 serum (GAM-B1C) was obtained from Nordic, Tilburg, The Netherlands. Anti-C4 serum (anti-Ss) was prepared as described earlier (16). The following materials were generous gifts: reverse transcriptase from avian myeloblastosis virus of Dr. J. Beard (National Cancer Institute, Bethesda, Md.); S1-nuclease of Dr. U. Schibler (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland); the plasmid pBR 322 and *Escherichia coli* strain K12-HB101 of Dr. W. Gehring (University of Basel, Switzerland); tetracycline (Achromycin) of Opopharma (Zürich). Ampicilline (Penbritin) was obtained from Beecham Research Laboratories. MeHgOH (water-soluble) was purchased from Alpha. [α-^{32}P]ATP and [γ-^{32}P]ATP were from the Radiochemical Centre Ltd, Amersham. Formamide (p.a., Merck) was recrystallized twice before use. Calf liver tRNA (Boehringer) and *E. coli* tRNA (Sigma) were extracted with phenol-chloroform prior to use. *Pst* I was prepared as was previously described (20).

Containment—All work involving recombinant DNA was carried out according to the National Institutes of Health guidelines for recombinant DNA. Recombinant plasmids were constructed under P3 physical, and EK1, biological containment (HB101, pBR 322) (National Institutes of Health guidelines of April 11, 1979).

Preparation of Partially Purified RNA—Polyadenylated RNA was prepared from fresh mouse livers by phenolchloroform extraction and oligo(dT) chromatography as described elsewhere (21) with the following modifications: 0.2 mg/ml of heparin was present during the homogenization and 0.5 mg/ml during the phenolchloroform extractions; the loading buffer for the oligo(dT)-cellulose column contained 0.3 M NaCl.

An average of 60 μg of polyadenylated RNA was recovered per liver, containing no detectable amounts of 28 S ribosomal RNA (Fig. 2A, position 5.1 kilobases). For size fractionation, the polyadenylated RNA was heated for 3 min at 68°C in 0.5% SDS,[1] chilled, and loaded on a linear 5 to 25% (w/w) sucrose gradient (12 ml) in a buffer

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§ Recipient of European Molecular Biology Organization Fellowship ALTF-93-1979.

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; base, (deoxy) ribonucleotide; DBM-paper, diazobenzyloxymethyl paper; MeHgOH, methylmercuryhydroxide; Pipes, 1,4-piperazinediethanesulfonic acid.

containing 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.4), 0.001 M EDTA, and 0.5% SDS. Between 0.4 and 0.6 mg of RNA were loaded on one gradient. After centrifugation for 4½ h at 40,000 rpm at 25°C in an SW 40 rotor (Beckman), the ≥28 S fraction (approximately 5% of the total) was collected and precipitated twice with ethanol.

Cell-free Translation—Cell-free translations were carried out using a commercial rabbit reticulocyte lysate (New England Nuclear) with [^{3}H]leucine or [^{35}S]methionine as radioactive precursors. Translation conditions were as specified by the supplier and essentially as previously described (22). Approximately 150 ng of mRNA were used/12.5-μl reaction.

Immunoprecipitation—For direct immunoprecipitation, translation reactions were diluted with 0.6 ml of detergent buffer, containing 0.15 M NaCl, 0.1 M Na-phosphate (pH 7.1), 0.02 M EDTA, 0.1% SDS, 1% Na-deoxycholate, 1% Nonidet P-40, and 0.1 mg/ml of phenylmethylsulfonyl fluoride. Fresh EDTA-plasma was prepared as described earlier (16). It was added as a source of carrier protein to immunoprecipitation reactions at equivalence with the antiserum: 5 μl of EDTA-plasma plus 150 μl of anti-C3 serum or 25 μl of EDTA-plasma plus 150 μl of anti-C4 serum. Precipitations were allowed to proceed overnight at 4°C. The immunoprecipitate was collected by centrifugation through a cushion of 1 M sucrose in detergent buffer for 10 min at 16,000 × *g*, washed once with detergent buffer, and twice with 0.15 M NaCl and 0.1 M Na-phosphate (pH 7.1).

SDS-Polyacrylamide Gel Electrophoresis—Discontinuous 7.5% gels and electrophoresis buffer were prepared according to an established procedure (23). Samples were boiled for 5 min in 5% SDS, 0.08 M Tris-HCl (pH 6.8), 9% glycerol, 0.002% bromphenol blue, and 5% dithiothreitol. After the electrophoresis the gels were treated with 50% (w/v) trichloroacetic acid, stained and destained. Fluorography was performed with En^{3}Hance (New England Nuclear). Radioactive bands were visualized on a preflashed film (24).

Molecular Cloning of cDNA—Double stranded cDNA was synthesized as described elsewhere (25). Twenty-five micrograms of size fractionated mRNA (≥28 S) was incubated in a 0.5-ml reaction mixture with 50 units of reverse transcriptase for the synthesis of the first strand and with 100 units for the synthesis of the second strand. Approximately 2 μg of S1-nuclease-resistant DNA were recovered and size-fractionated by centrifugation in a sucrose gradient. Fractions containing DNA of approximately 2000 to 2500 and 2500 to 6000 basepairs were pooled and precipitated twice with ethanol. Homopolymeric extensions were added to the cDNA using terminal transferase (Bethesda Research Laboratories, Inc.) as described elsewhere (26). cDNA (0.2 μg) was incubated in a 200-μl reaction with 10 units of terminal transferase at 37°C for 10 s, in order to reach a tail length of 8 to 30 (dC) per 3′-end. After phenolchloroform extraction, the material was precipitated twice with ethanol. pBR 322 DNA was digested with *Pst* I, linear molecules were selected and elongated with $(dG)_{10}$ tails. Equimolar amounts of vector and cDNA (200 ng in total) were annealed in 0.6 ml of 0.1 M NaCl, 10 mM Tris-HCl (pH 7.5), and 0.2 mM EDTA.[2] *E. coli* cells (strain K12-HB101) were transformed with 200 ng of this DNA mixture as described previously (27). 310 ampicilline-sensitive and tetracycline-resistant colonies were isolated. Colony hybridizations were carried out as described elsewhere (28) using gel eluted RNA as probe. Gel-eluted RNA (see below) was dissolved in 0.1 M NaOH and incubated for 30 min on ice, neutralized, and precipitated with ethanol. End-labeling was performed with [γ-^{32}P]ATP using T4-polynucleotide kinase (P-L Biochemicals, Ref. 29). Candidate clones were grown on a preparative scale, plasmids were amplified with chloramphenicol (0.2 mg/ml), and DNA was prepared by the cleared lysate procedure followed by banding in a CsCl gradient containing ethidiumbromide as previously described (30).

Gel Electrophoresis and Gel Elution of RNA—mRNA was size-fractionated by electrophoresis in 1% agarose gels containing 5 mM MeHgOH as described previously (31). For elution of RNA, 250 μg of polyadenylated RNA were loaded on a preparative slot (50 × 4 × 1 mm^3). Electrophoresis proceeded for 40 h at 1.5 V/cm. Side strips were stained with ethidium bromide and RNA bands were localized by photography under UV light. RNA bands were excised from the preparative gel according to the stained pattern. RNA was recovered from the gel slices as described elsewhere (21) except that 0.5 M LiCl was replaced by 0.5 M ammonium acetate in order to fix the MeHgOH.

For analytical hybridizations RNA was separated in the same way, using 50 μg of RNA in a slot of 5-cm width. After electrophoresis, the RNA was transferred onto DBM-paper as previously described (32) except that the transfer medium was buffered with 25 mM K-phosphate (pH 6.5).

Gel Electrophoresis and Gel Elution of DNA—DNA restriction fragments were separated by electrophoresis in horizontal 1% agarose gels in 40 mM Tris-HCl, pH 7.8, 5 mM Na-acetate, and 1 mM EDTA.

For hybridization purposes the DNA was transferred onto nitrocellulose sheets as described elsewhere (33). Preparative elution of DNA fragments from agarose was performed as described previously.[3]

Hybridizations—RNA, which was immobilized on DBM-paper (Northern blot) was hybridized with nick-translated (34) recombinant DNA probes as described elsewhere (32).

Plasmids, carrying cDNA inserts, were immobilized on DBM-paper and used for preparative hybridization with RNA, essentially as previously described (35). Eight sets of two filters of 11 mm in diameter, carrying each 10 to 20 μg of linearized single-stranded plasmid DNA, were prehybridized in 4 ml of 50% (v/v) formamide, 0.6 M NaCl, 15 mM Pipes (pH 6.4), 0.2% SDS, 100 μg/ml of poly(U), and 1 mg/ml of calf liver tRNA for 30 min at 41°C. The main hybridization contained all filters in 0.8 ml of 50% (v/v) formamide, 0.6 M NaCl, 1.5 mM EDTA, 0.2% SDS, 100 μg/ml of poly(U), 250 μg/ml of calf liver tRNA, and approximately 100 μg of size fractionated mRNA (≥28 S). After a hybridization period of 22 h at 41°C, the filters were washed batchwise 6 times for 10 min at 41°C with 20 ml of 50% (v/v) formamide, 45 mM NaCl, 4.5 mM Na-citrate, 20 mM Pipes (pH 6.4), 1 mM EDTA, and 0.2% SDS. Hybridized RNA was eluted from each set of filters by four subsequent incubations at 65°C in 100 μl of preheated 80% (v/v) formamide, 0.1% SDS, 10 mM Pipes (pH 6.4), and 20 μg/ml of *E. coli* tRNA, precipitated three times with ethanol and translated *in vitro*.

Hybridization of DNA immobilized on nitrocellulose sheets was performed as described elsewhere (33).

RESULTS

Demonstration of the Presence of the mRNAs for pro-C3 and pro-C4 in Mouse Liver—In order to show that the liver is one of the sites of synthesis of C3 and C4 in the mouse, we translated total mouse liver mRNA *in vitro* (Fig. 1*A*, *Track 4*) and performed immunoprecipitations on the products with antibodies specific for C3 and C4. These immunoprecipitations provided indications for the synthesis of C3 and C4 (data not shown). Because of the relatively low translational efficiency of high molecular weight mRNAs we fractionated the total mRNA prior to translation. Translation products specified by the ≥28 S fraction are shown in Fig. 1*A*, *Track 5*. The presence of pro-C3 and pro-C4 (Fig. 1*A*, *Tracks 2* and *3*, indicated by *arrows*) was demonstrated by direct immunoprecipitations on a 10-fold larger sample, to which unlabeled C3 and C4 (from plasma) were added at equivalence with the antisera. The characteristic pattern of subunits of plasma C3 and C4 after staining the proteins is shown in Fig. 1*B*, indicated by *arrows*. The C3 α subunit can clearly be seen (Fig. 1*B*, *Tracks 2* and *7*), whereas most of the C3 β subunit disappeared, probably due to proteolytic cleavage giving rise to the band C_3x. The α, β, and γ subunits of C4 and Slp can all be seen (Fig. 1*B*, *Tracks 3* and *8*). This pattern of stained immunoprecipitated proteins served as internal control for the high specificity of the immunoprecipitation. The specificity of the goat anti-mouse-C3 serum was further confirmed by our observation that a rabbit anti-rat-C3 serum precipitated the same pattern of nonradioactive proteins from mouse plasma (data not shown).

We determined for pro-C3 a size of approximately M_r = 175,000 and for pro-C4 of M_r = 190,000 (Fig. 1*A*, *Tracks 2* and *3*). Proteins of lower molecular weight were also specifically immunoprecipitated. They represent probably prematurely terminated polpeptides. No products of sizes similar to those of pro-C3 and pro-C4 were detected upon translation of fractions of RNA sedimenting slower than 28 S (data not shown).

[2] According to Rowekamp and Firtel, personal communication.

[3] McMaster, G. K., Beard, P., Engers, H., and Hirt, B. (1980) *Virology*, in press.

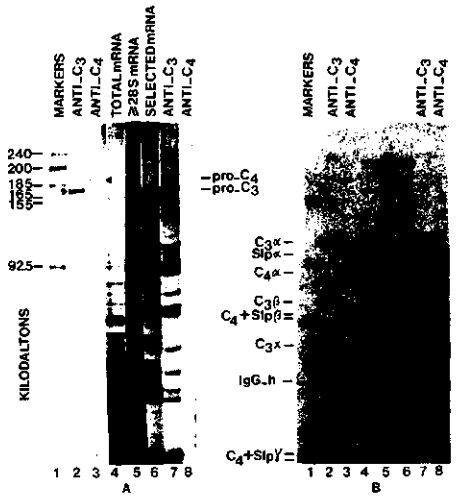

FIG. 1. **SDS-polyacrylamide gel electrophoresis of translation products.** *A*, mRNA samples were translated *in vitro* and their products were analyzed directly or after immunoprecipitation by SDS-polyacrylamide gel electrophoresis. Three microliters of the total translation products and all of the immunoprecipitate were loaded on each track. *Track 1*, molecular weight markers: radioactive: 240,000 filamin, 200,000 myosin, 185,000 macroglobulin, 92,500 phosphorylase B (New England Nuclear); from the stained gel: RNA polymerase at 165,000 and 155,000. *Track 2*, direct immunoprecipitation with anti-C3 serum from 28 μl of the assay in *Track 5*. *Track 3*, as *Track 2*, but with anti-C4 serum (exposure time 3-fold longer as for the other tracks). *Track 4*, 150 ng of unfractionated mRNA were translated in a 125-μl assay. *Track 5*, 450 ng of size-fractionated (≥28 S) RNA were translated in a 60-μl assay. *Track 6*, translation products of RNA which was selected by hybridization with a recombinant plasmid containing C3-related sequences (pMLC-3) translated in a 60-μl assay. *Track 7*, direct immunoprecipitation with anti-C3 serum from 28 μl of the same assay as in *Track 6*. *Track 8*, as *Track 7*, but with anti-C4 serum. *B*, the same gel but stained with Coomassie brilliant blue.

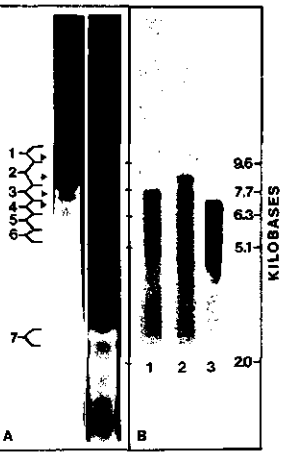

FIG. 2. **Agarose gel electrophoresis of RNA.** RNA was separated by electrophoresis in denaturing agarose gels containing 5 mM MeHgOH (31). Electrophoresis was for 40 h at 1.5 V/cm. *A*, 250 μg of mRNA were loaded on a preparative gel. A side strip was stained and photographed (two exposures are shown). Regions indicated with *1* to *7* were excised from the corresponding gel and the RNA was eluted (see text). The *arrows* indicate individual bands which could be detected on the original picture. *B*, 50 μg of mRNA were loaded in a slot of 5-cm width and electrophoresed as under *A* on a parallel gel. The RNA was transferred to and bound on DBM-paper (32). Individual strips of the paper were hybridized with nick-translated recombinant plasmids. *Strip 1*, recombinant plasmid containing C3-related sequences (pMLC3-1); *Strip 2*, plasmid containing sequences related to a 165,000-dalton protein (non C3-related); *Strip 3*, a plasmid containing sequences coding for an unidentified protein. The size calibration of the mRNAs was achieved using ribosomal RNA and its precursors. Their sizes are given in kilobases: 18 S = 2.0, 28 S = 5.1, 32 S = 6.3, 36 S = 7.7, and 41 S = 9.6 (37).

Size Determination of the mRNAs for C3 and C4—In order to determine the sizes of the mRNAs for C3 and C4, we eluted mRNA from slices of a denaturing MeHgOH agarose gel (31). Several discrete bands of RNA species migrating slower than 28 S ribosomal RNA (5.1 kilobases) could be distinguished on the original picture (indicated by *arrows* in Fig. 2*A*). After elution, the RNA was divided into two aliquots. One was used for colony hybridization(see below) and the other was translated *in vitro* (Fig. 3). *Track T* represents translation products of unfractionated mRNA, *Tracks 1* to *6* represent the products of the RNA eluted from the Slices 1 to 6, respectively (Fig. 2*A*). Translation products with the same apparent molecular weight as pro-C4 (190,000) and pro-C3 (175,000), indicated by *arrows*, were mainly specified by RNA eluted from *Slices 2* (~8.4 kilobases) and *3* (~7.5 kilobases). Therefore, it is likely that the mRNAs for C3 and C4 contain approximately 7.5 and 8.4 kilobases. Too small amounts of eluted RNA were available to perform immunoprecipitations on the cell-free translation products, but the size estimation for the mRNA for C3 was confirmed by hybridization data (Fig. 2*B*, see below).

Characterization of Recombinant Plasmids containing cDNA—We followed a general cloning procedure which has been previously described (36). Double-stranded cDNA was inserted into the *Pst* I restriction site of the plasmid pBR 322 and recombinant molecules were used to transform *E. coli* strain HB101. Identification of the plasmids was achieved in two steps: 1) colony hybridization (28) with a specific RNA probe; 2) hybrid-selected translation (35) followed by immunoprecipitation.

For the colony hybridization we used RNA which was eluted from slices of a gel (see above, Fig. 2*A*) as probe. Because it was clear from Fig. 3 that more than one abundant mRNA species was present in each of the RNA samples eluted from the Slices 2 and 3 (Fig. 2*A*), we performed a multiple screening using several probes. The DNA of 20 colonies hybridized significantly with one or more of the RNA probes from Slices 2, 3, and 4 (Fig. 2*A*), but none hybridized with the control RNA from Slice 7 (data not shown). Plasmid DNAs which hybridized only with the probe from Slice 3 were classified as candidates for C3 and others hybridizing with both the probes from the Slices 2 and 3 were classified as candidates for C4.

In order to establish the coding specificity of the candidate plasmids, denatured DNA was immobilized on DBM-paper and used to select specific and translatable RNA (35). Three recombinant plasmids were found to retain RNA which directed cell-free synthesis of proteins which were specifically immunoprecipitable with antibodies directed against C3. One example is shown in Fig. 1*A*, *Track 6*. A major translation product migrated at the same position as pro-C3 (M_r = 175,000), but many proteins with a lower molecular weight were observed. Immunoprecipitation with anti-C3 serum (Fig.

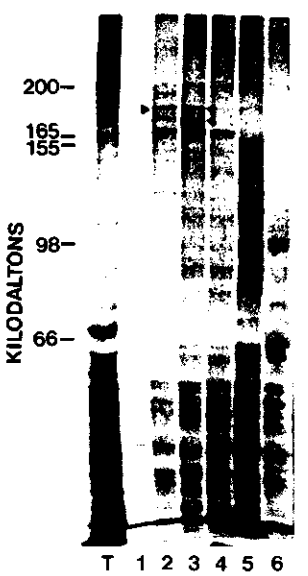

FIG. 3. **SDS-polyacrylamide electrophoresis of translation products of gel-eluted RNA.** RNA was eluted from the gel slices which are indicated in Fig. 2*A*. The eluted mRNA was translated *in vitro* in 12.5-μl assays. Entire samples of translation products were analyzed by SDS-polyacrylamide gel electrophoresis. *Track T*, unfractionated mRNA (100 ng in 12.5 μl). *Tracks 1* to *6*, mRNA eluted from the Slices 1 to 6, respectively, in Fig. 2*A*. Molecular weight calibration: RNA polymerase, 165,000, 155,000; phosphorylase *a*, 98,000; bovine serum albumin, 68,000. The position of 200,000 was obtained by comparison of the predominant protein bands of the reticulocyte lysate with the calibration in Fig. 1.

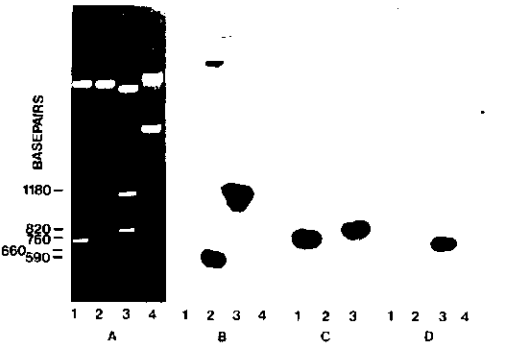

FIG. 4. **Restriction enzyme analysis of plasmids containing C3-related sequences.** The DNA of recombinant plasmids pMLC3-2 (*Track 1*), pMLC3-3 (*Track 2*), pMLC3-1 (*Track 3*), and an unrelated plasmid (*Track 4*), each 300 ng, were digested with *Pst I* restriction enzyme. Fragments were separated by electrophoresis in a 1% agarose gel. As size markers were used: phage λ DNA digested with *Eco* RI and pBR 322 DNA digested with *Alu* I (data not shown). After staining the DNA with ethidium bromide a picture was taken (*Panel A*) and the DNA was transferred to nitrocellulose sheets and subsequently hybridized with nick-translated *Pst* I restriction fragments of the insert of pMLC3-1 (see text): *Panel B*, 1180-base pair fragment, *Panel C*, 820-base pair fragment; and *Panel D*, 660-base pair fragment.

1*A*, *Track 7*) and anti-C4 serum (Fig. 1*A*, *Track 8*) showed that not only the M_r = 175,000 protein but also the majority of the proteins of lower molecular weight were related to C3. The last are probably due to fragmentation of the mRNA during the hybridization. Again the pattern of stained proteins (Fig. 1*B*, *Tracks 7* and *8*) showed an efficient immunoprecipitation of high specificity.

A restriction analysis with *Pst* I of the plasmids containing sequences complementary to mRNA for C3 is shown in Fig. 4*A*, *Tracks 1*, *2*, and *3*. The sizes of the inserts and *Pst* I fragments of the inserts were determined using phage λ DNA digested with *Eco* RI and pBR 322-DNA digested with *Alu* I as markers (data not shown). The inserts of the three C3 plasmids measure 760 base pairs (pMLC3-2, Fig. 4*A*, *Track 1*), 590 base pairs (pMLC3-3, Fig. 4*A*, *Track 2*), and 2900 base pairs (pMLC3-1, Fig. 4*A*, *Track 3*). The insert of pMLC3-1 contains three internal *Pst* I restriction sites, giving rise to fragments of the following approximate lengths: 1180, 720, 660, and 140 base pairs (the latter one is too small to be seen in Fig. 4*A*, *Track 3*).

In order to determine whether the three clones contain (partially) overlapping sequences, we examined their ability to cross-hybridize. *Pst* I restriction fragments of each plasmid and of a control non C3-related plasmid (Fig. 4*A*, *Track 4*) were separated by electrophoresis in an agarose gel and the DNA transferred to nitrocellulose sheets. The DNA was hybridized to each of the three largest *Pst* I restriction fragments of the insert of pMLC3-1. These fragments were isolated by elution from an agarose gel and were rendered radioactive by nick translation. In Fig. 4, one can see that the 1180-base pair fragment hybridizes to the insert of pMLC3-3 and to itself (Fig. 4*B*, *Tracks 2* and *3*), the 820-base pair fragment hybridizes to the insert of pMLC-2 and to itself (Fig. 4*C*, *Tracks 1* and *3*), and that the 660-base pair fragment hybridized only to itself (Fig. 4*D*, *Track 3*). None of the fragments hybridized significantly with the unrelated plasmid (Fig. 4, *B* and *D*, *Track 4*).

In order to confirm the size determination of the mRNA for C3 (see above), mouse liver mRNA was fractionated by electrophoresis in a denaturing gel, transferred to DBM-paper, and hybridized with nick-translated plasmid DNA, containing C3 sequences (Fig. 2*B*, *Strip 1*). The hybridizing RNA species was determined to contain indeed approximately 7,500 bases. As a specificity control RNA on two other strips was hybridized with plasmid DNA which did not contain C3 sequences (Fig. 2*B*, *Strips 2* and *3*).

DISCUSSION

The cell-free translation results show that the primary translation products for mouse C3 and C4 proteins are large single chain polypeptides and that the subunits are not translated from separate, smaller mRNA species. No translation products of the size of mature subunits were detectable by immunoprecipitation. This result confirms observations of other authors for pro-C3 and pro-C4 molecules in other species (12–14) and shows that not only macrophages (15, 16) but also liver cells of mice produce C3 and C4 as single chain precursors. Our findings confirm a preliminary report for the presence of C3 and C4 mRNA species in mouse liver (38). However we do not observe any proteolytic processing into fragments of the size of mature subunits in the cell-free system as described by these authors. Upon prolonged exposure of autoradiographs of immunoprecipitations of pro-C3 and pro-C4 after cell-free translation, a characteristic pattern of lower

olecular weight polypeptides was observed several times data not shown). The same pattern was later observed much ore clearly when mRNA was translated *in vitro* after hybrid election. These proteins of lower molecular weight may rep-esent early termination polypeptides, due to fragmentation f the mRNA and premature release of nascent protein from he polyribosomes.

Gel eluted RNAs of the 7.5-kilobase size class (Fig. 2*A*, *legion 3*) and the 8.4-kilobase size class (Fig. 2*A*, *Region 2*) ere translatable into polypeptides migrating with the same obility as pro-C3 and pro-C4, respectively. Also in this xperiment (Fig. 3), a background of low molecular weight anslation products is visible, which is not likely to be due to ontaminating gel-eluted smaller mRNAs. Therefore, prema-ure termination appears to be a frequent event during the anslation of very large mRNAs in reticulocyte lysates under tandard conditions. As the reticulocyte lysate is incapable of roperly modifying nascent polypeptides, it is unlikely that he pro-C3 and pro-C4 molecules which we have described ere, are fully processed. We observed (data not shown) that vith respect to a set of molecular weight standards the cell-ree translated pro-C3 and pro-C4 migrate faster in gel elec-rophoresis than the intracellular precursors from macro-hages, which were metabolically labeled. We should there-ore designate the cell-free translation products pre-pro-C3 nd pre-pro-C4. However, we do not want to introduce this omenclature as long as the physical differences between the ell-free translation products and the functional intracellular recursors are not characterized.

The finding that the incompletely processed cell-free trans-ation products can be immunoprecipitated with heterologous ntisera raised against the serum C3 and C4 molecules indi-ates that there are common antigenic determinants between hese components. This finding was essential for the later dentification of the cDNA clones.

By *in vitro* translation it is impossible to estimate the bundancies of the mRNAs for C3 and C4 for two reasons. First, we do not know how efficient the immunoprecipitation f the products of *in vitro* translation is. Second, we observed y titrating the optimal amount of mRNA to be used per ranslation assay, that the high molecular weight RNA species re translated *in vitro* with a lower efficiency than RNAs of ow molecular weight (data not shown).

Because none of the recombinant plasmids hybridized sig-ificantly (data not shown) with the RNA probe of low molecular weight (Fig. 2*A*, *slice 7*), we conclude that the size fractionation of the mRNA prior to the molecular cloning was efficient. Screening of the cDNA clones by colony hybridiza-tion and hybrid-promoted translation revealed three different plasmids containing cDNA inserts with sequence homology to the mRNA for C3. The largest insert, 2900 base pairs (pMLC3-1), cross-hybridizes with the two smaller inserts, pMLC3-2 and pMLC3-3. Since no protein sequence data for mouse C3 are available for comparison it is at present not easily possible to prove that the cDNA inserts of the plasmids which are described here are complementary in their entire length to mRNA for C3. Despite the recovery of three independent clones complementary to portions of mRNA for C3, a remote possibility remains that these plasmids carry cDNA sequences homologous to a C3-related but not identical mRNA of low abundancy.

So far we have not identified any plasmid with a cDNA insert corresponding to the mRNA for C4. This may be an indication that the mRNA for C4 is less abundant than mRNA for C3. A fraction of the cell-free translated proteins with a molecular weight of 190,000 may not be related to C4, thus causing a wrong estimation of the abundancy of mRNA for C4. In this respect, it should also be noted that the experiment in which mRNA was eluted from gel slices (Fig. 2*A*) showed that RNAs eluted from the slices of the 7.5- and 8.4-kilobase region were both translatable into a product of M_r = 190,000 (Fig. 3). Therefore, there may be two or more mRNA species coding for proteins of the same molecular weight.

The final determination of the size of the mRNA for C3 relies on the argument that a large majority of the translation products of the hybrid-selected mRNA are C3-related (Fig. 1, *Tracks 6* and *7*). Therefore, the major mRNA species with which these cDNA plasmids hybridize on a Northern blot should be the mRNA for C3. Only one hybridizing species with a size of 7,500 bases was found (Fig. 2*B*, *Track 1*).

For a primary translation product of M_r = 175,000, one expects a coding sequence of at most 5,300 bases. Therefore, the mRNA for C3 contains at least 2,000 bases in non-translated regions, more than one-quarter of its whole length. It will be of interest in the future to investigate the structural organization of the mRNA for C3 and its gene in more detail.

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4 EXPRESSION OF THE GENE FOR THE THIRD COMPONENT OF THE COMPLEMENT SYSTEM (C3) IN PRIMARY MACROPHAGES AND CELL LINES AND THE EFFECT OF THE GLUCOCORTICOID HORMONE DEXAMETHASONE*

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Summary

Synthesis of C3-protein was observed in primary mouse macrophages and in several tissue culture cell lines of mouse macrophage (J774.2 and IC21) and rat hepatic origin (HTC and FaO) by metabolic labeling, immunoprecipitation and electrophoresis in SDS-polyacrylamide gels. After starch induction *in vivo* of macrophages a decrease of C3- and C4-protein biosynthesis was observed *in vitro*, with respect to total protein synthesis. In two cell lines (HTC and J774.2) the synthesis of C3-protein could be decreased approximately two-fold by the addition of the glucocorticoid hormone dexamethasone to the medium. This decrease was observed for both pro-C3 and mature secreted C3 and was dose dependent. The decrease occurred between 10^{-7} and 10^{-8} M dexamethasone. In contrast the C3-mRNA concentrations in J774.2 cells were increased approximately 1.5-fold after the addition of dexamethasone.

Introduction

An important defence mechanism against infections is mediated through the activation of the complement system in blood plasma and tissue fluids of vertebrates. After this activation, the clearance of foreign and own material (bacteria, viruses, aged cells etc.) is achieved by cell lysis and phagocytosis of particles (for review see 1,2). The third component of complement (C3) plays an essential role in both these events. In this paper we want to report on studies on the regulation of the biosynthesis of C3.

Hepatocytes are the major producers of C3 and of many of the other complement components. In addition peritoneal macrophages and a few other cell types are known to synthesize C3 (for review see 3,4). For the study of the C3-gene expression it is advantageous to work with cultures of cells which express the

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C3-gene and which respond faithfully to regulatory stimuli. Therefore we first investigated the biosynthesis of C3-protein by primary peritoneal macrophages in tissue culture and asked wether the mouse macrophage derived cell lines IC21 (5) and J774.2 (6) and the rat hepatoma lines HTC (7) and FaO (8) synthesized C3. Thereafter we studied the effect of various stimuli on the capacity of the cells to synthesize C3-protein and in one case we compared the effect on the C3-protei synthesis with the effect on C3-mRNA concentrations, using a cloned cDNA hybridi zation probe specific for C3 (9). One of the stimuli used was the intraperitonea injection of starch, three days before collection of the peritoneal cells. This treatment mimics a localized inflammation and has been reported (10,11) to affec the synthesis of several hemolytically active complement components by guinea pi macrophages. Furthermore we tested the effect of dexamethasone, a potent anti-inflammatory agent, on the C3-synthesis in several cell lines. It has been repor ted (12) that hydrocortisone enhances up to nine-fold the synthesis of hemolytically active C3 in a rat hepatoma line. In macrophage derived cell lines we want ed to determine, wether the C3-expression is repressed by the addition of dexamethasone, as has been reported for several macrophage secretion products,eg. collagenase,elastase, plasminogen activator (13-16), or wether it responded in a different way.

Experimental procedures

Animals and primary cell cultures

DBA/2J male mice are carried in the breeding facility of our institute. Primary cultures of peritoneal exudate cells from four animals per tissue culture dish (Ø 9cm) were prepared as previously described (17). In order to mimic an inflammation mice were treated intraperitoneally with 3 ml of 2% starch in endotoxin free physiological salt solution, three days prior to the collection of the cells. The peritoneal cells from these mice are refered to as induced macrophages. Usually 2-3 X 10^6 cells, containing 20-30% macrophages were obtained from control mice and 20-30 X 10^6 cells containing 60-70% macrophages from starch induced mice[1].

Cell lines

The macrophage derived cell lines IC21 (5) and J774.2 (6) were obtained from Drs. H.Engers and J.Mauel (Epalinges, Switzerland) and Dr. B.Bloom (New York, USA) respectively. The first are macrophages which are transformed by SV40 virus, the second derive from a natural reticulo-sarcoma. The rat hepatoma lines HTC (7) and FaO (8) were obtained from Drs. K.Yamamoto (San Francisco, USA) and M.Weiss (Gif sur Yvette, France) respectively. In all experiments Dulbeccos modified

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1) Dr J.Mauel, Epalinges, Switzerland, personal communication.

Eagles medium supplemented with 10% heat inactivated fetal bovine serum was used. The medium for the J774.2 cells was supplemented with 0.2 mg/ml of L-glutamine. If dexamethasone was used, the hormone was added from a 10^{-3} M solution in ethanol.

Radioactive labeling and immunoprecipitation of proteins

Cell cultures were washed with methionine free medium and 50-120 µCi of ^{35}S-methionine (10^3 Ci/mmole) was added in 1.5 ml of the same medium. Unless otherwise specified, labeling was for 1.5 hrs, followed by a chase for 1.5 hrs after the addition of unlabeled methionine to a final concentration of 300 µM. Tissue culture supernatants were collected, centrifuged in order to remove floating cells and made 10 mM in N-aethylmaleinimid. Cytoplasmic extracts were prepared as described elsewhere (18). The proteins from 10 µl of extract were precipitated in 10% TCA[2)] and the amount of radioactivity was determined as an estimate of the total amount of newly synthesized protein. In some cases (indicated in the text) the tissue culture supernatants were passed three times over a column with 3 ml of gelatin-sepharose (kind gift of Dr. L.Zardi, Epalinges, Switzerland), in order to remove fibronectin (19). The amount of bound fibronectin was determined after washing the column with 4 M NaCl and elution with 3 M urea (19). Samples were counted in aquasol (New England Nuclear Inc.) after a 5-fold dilution in water. Indirect immunoprecipitation with antibodies and Staphylococcus aureus and the SDS-polyacrylamide gel electrophoresis were as described earlier (17), except for the presence of 1% Na-deoxycholate and 0.1% SDS during the immunoprecipitation. The amount of label in individual protein bands was measured by dissolving gel slices in 0.25 ml of 30% H_2O_2 for 6-8 hrs at 80°C and counting in aquasol. Goat anti mouse B1C serum (anti-C3 serum) was obtained from Nordic, Tilburg, the Netherlands. The preparation of anti mouse C4 serum is described elsewhere (17).

RNA techniques

Total cellular RNA from tissue culture cells was prepared by extraction with hot phenol (20). Extraction of total cytoplasmic RNA from mouse liver and the isolation of polyadenylated RNA were performed as described elsewhere (21) with minor modifications (9). RNA samples were denatured in glyoxal (22) for 15 min at 50°C in presence of 0.2% SDS and electrophoresed through an 0.7% agarose gel in 10 mM Na-phosphate, pH 6.5, for 70 min at 70 mA and 10 V/cm. The RNA was transferred onto nitrocellulose filters (23) and hybridized to a radioactive C3 specific DNA probe as described (9) except for the omission of the first prehybridization step.

Results

Biosynthesis of C3-protein by primary macrophages and cell lines

The biosynthesis of of C3-protein by cells of different origen was studied by metabolic labeling of proteins with ^{35}S-methionine followed by indirect immunoprecipitation and polyacrylamide electrophoresis of the immunoprecipitates. After a pulse labeling for 1.5 hrs and a chase period of 1.5 hrs in presence of an

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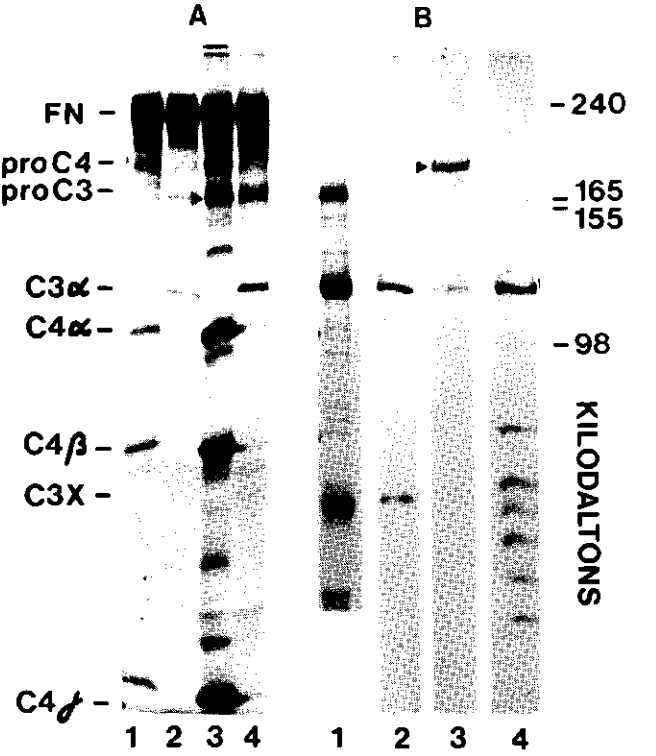
2) the abbreviations used are TCA, trichloroacetic acid; SDS, sodium dodecyl sulphate

excess of unlabeled methionine, indirect immunoprecipitations were performed on cytoplasmic extracts and tissue culture supernatants, using Staphylococcus aureu to precipitate immune complexes. In the experiments with primary macrophages the immunoprecipitates were contaminated by fibronectin (FN in fig. 1A) which has a strong affinity for Staphylococcus aureus. A protein with a molecular weight of 200'000 contaminated the immunoprecipitates from HTC cells (arrow in fig. 1B, track 3).

After the 1.5 hrs chase period most of the immunoprecipitable radioactive C3 related material was exported from the cytoplasm into the tissue culture supe natant for both primary macrophages and HTC cells (data not shown). For routine purposes therefore only supernatants were examined for the presence of C3-protei The following molecular weights for C3 specific immunoprecipitated polypeptides were observed: pro-C3 175'000, C3α 120'000, C3β 75'000 (not shown) and C3X 50'000. The last polypeptide probably is a breakdown product of C3β, as we obser ved a reciprocal relationship between the abundancies of C3β and C3X in several experiments (data not shown).

The syntheses of C3-and C4-proteins by starch induced and resident primary mouse macrophages were compared (fig. 1A). In these experiments total peritoneal exudate cell populations were labeled, but it is known that only the adherent macrophages are responsible for the synthesis of C3 and C4 protein (17). As show in fig. 1A starch induced macrophages synthesize and secrete less C4 (α,β and γ chains) and less C3 (α chain), tracks 1 and 2 resp., than resident macrophages, tracks 3 and 4. Counting of the radioactivity contained in the individual protei bands showed a 20-fold decrease for C4 and a 5-fold decrease for C3. In these experiments the amounts of tissue culture supernatants used for immunoprecipita- tion were not the same. The amounts taken were normalized in such a way that the represented the same quantity of newly synthesized intracellular protein. There- fore we compared the synthesis of C4 and C3 with the total protein synthesis (see also the discussion). We cannot distinguish whether the reduced synthesis of C4 and C3 was due to a decrease in the fraction of complement secreting cells, or that all cells produced lower amounts of C3 and C4. The strong band, indicated by an arrow in fig. 1A, track 3, containing polypeptides with a molecular weight of 175'000, represents probably an intermediate processing product of pro-C4. The sum of $\alpha + \beta$ chains could account for 175'000 dalton, what would be in agreement with the proposed α-β-γ order of polypeptide chains within pro-C4 (24).

Two different mouse macrophage derived cell lines, IC21 and J774.2 (fig. 1B,



igure 1. Biosynthesis of C3 and C4 protein by primary macrophages and of C3 protein by continuous cell lines

ells were metabolically labeled with ^{35}S-methionine. Tissue culture supernatants f approximately one dish (Ø 9cm) were used for immunoprecipitation. Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophresis. A. Indirect immunoprecipitates from primary peritoneal macrophages: track 1 induced cells using nti-C4, track 2 same using anti-C3, track 3 resident cells using anti-C4 and rack 4 same using anti-C3. B. Cell lines: track 1 direct immunoprecipitate using nti-C3 (9) from IC21 cells, track 2 indirect immunoprecipitate using anti-C3 rom J774.2 cells, track 3 same from HTC cells and track 4 same from FaO cells.

racks 1 and 2) and two different rat hepatoma lines, HTC and FaO (fig. 1B, racks 3 and 4) all synthesized and secreted C3-protein. Polypeptide chains with olecular weights characteristic for C3 were detected after immunoprecipitation: 3α (fig. 1B, tracks 1-4), C3X (tracks 1,2) and pro-C3 (track 1) from the tissue-ulture supernatants and pro-C3 from the cytoplasmic extracts (observed only fter short pulse labelings; for J774.2 see fig. 2, track 2, for the others the ata are not shown). The polypeptide of 200'000 dalton, indicated by an arrow in ig. 1B, track 3, is probably not related to C3, as it sticks to Staphylococcus

TABLE 1. INHIBITION BY DEXAMETHASONE OF THE C3-BIOSYNTHESIS

Cell type	Dexamethasone 10^{-6} M	C3α a) cpm/band	Inhibition % of control	Fibronectin cpm b)	Stimulation of fibronectin % of control
J774.2	+	8215	--	--	
	-	3480	58	--	
HTC					
exp.1	+	200	--	22800	--
	-	95	52	69830	210
exp.2	+	510	--	69600	--
	-	240	52	195000	280
FaO	+	9297	--	--	
	-	7512	17	--	

a) Cells were metabolically labeled with ^{35}S-methionine. Indirect immunoprecipitations using anti-C3 were performed on tissue culture supernatants after depletion of fibronectin (see experimental procedures). Immunoprecipitates were submitted to SDS-polyacrylamide electrophoresis and the C3α bands were excised from the gel, solubilised and counted. The values are normalised with respect to the total amount of radioactivity incorporated into protein. One dish (Ø 9cm) was used per experimental point. b) Fibronectin was absorbed on a gelatin-sepharose column and subsequently eluted (see experimental procedures).

ureus even in the absence of antibodies. Using the supernatant from the FaO line fig. 1B, track 4), a series of small polypeptides was immunoprecipitated, the rigin of which was not investigated any further. The samples used for the immunorecipitations shown in fig. 1B, tracks 2, 3 and 4, were depleted from fibronecin before the immunoprecipitation (see experimental procedures). In tissue culure supernatants of HTC cells (fig. 1B, track 3) fibronectin represented 300-old more radioactive protein than C3, whereas the FaO and J774.2 cells only produced neglegible amounts of fibronectin. The IC21 cells were not tested for the resence of fibronectin and the immunoprecipitation shown in fig. 1B, track 1, as obtained in an independant experiment by direct immunoprecipitation (9).

ffect of dexamethasone on the synthesis of C3-protein

he effect of the synthetic glucocorticoid hormone dexamethasone on the biosynhesis of C3-protein was tested on HTC, FaO and J774.2 cells. Dense cultures were aintained in the absence or the presence of 10^{-6} M dexamethasone for 24-48 hrs rior to and during metabolic labeling. The synthesis of C3-protein was detected s described above and the amounts of radioactivity contained in the C3 band fter immunoprecipitation and gel electrophoresis were measured (table 1). For he HTC cells the amount of newly synthesized fibronectin also was determined see experimental procedures). From table 1 it is clear that the amount of newly ynthesized C3 is reduced by approximately two-fold in presence of 10^{-6} M dexaethasone in the HTC and J774.2 cells, whereas in the FaO cells only a minor derease was observed. In contrast the amount of fibronectin synthesized by HTC ells increased two to three-fold after addition of 10^{-6}M dexamethasone. The synhesis of C3 by J774.2 cells in respons to different hormone concentrations was lso followed (fig.2, tracks 3-8). Dense cultures were maintained for 48 hrs after ddition of increasing amounts of dexamethasone and subsequently labeled. One ulture was incubated with 10^{-6} M dexamethasone for 24 hrs (long enough to cause two-fold decrease of the C3 synthesis), followed by another 24 hrs without horione (fig. 2, track 9). Furthermore the effect of 10^{-6}M dexamethasone on the synhesis of intracellular pro-C3 was tested by pulse labeling cells for 30 min followed by immunoprecipitation from the cytoplasmic extract (fig. 2, tracks 1 and 2) he quantative evaluation of the experiment is shown in table 2. The results show hat the newly synthesized intracellular pro-C3 and extracellular mature C3 are oth reduced approximately two-fold by 10^{-6} M dexamethasone compared to control ultures (no hormone). The reduction occurs in the concentration range between

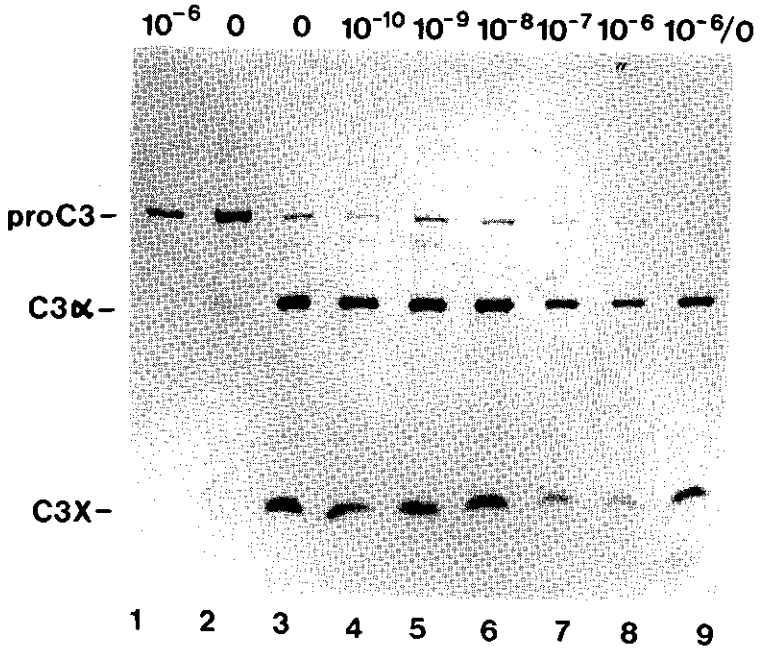


Figure 2. Response of the C3-protein biosynthesis to dexamethasone in J774.2 cel

J774.2 cells were incubated for 48 hrs in the presence of increasing amounts of dexamethasone and subsequently metabolically labeled with ^{35}S-methionine. Indirect immunoprecipitations using anti-C3 were performed from total cytoplasmic extracts (tracks 1 and 2) and from total tissue culture supernatants (tracks 3-9 One culture dish (Ø 6cm) was used per point. The molar concentrations of dexamethasone are indicated above the tracks. The cells used for track 9 were first incubated for 24 hrs in presence of hormone and thereafter for 24 hrs without hormone.

10^{-8} and 10^{-7} M dexamethasone (fig.2, tracks 6 and 7). The effect of the hormone is at least partially reversible because withdrawal of the hormone and a 24 hrs recovery period an increase in the synthesis of C3 was observed (fig. 2, track 9

The observation that 10^{-6} M dexamethasone has the same effect on the synthesis of pro-C3 and the appearance of mature C3-protein in the tissue culture supernatant indicates that the hormone probably does not act on the level of post-translational processing and secretion, but rather on the level of translation, mRNA synthesis or mRNA degradation.

Titration of C3-mRNA

In order to determine wether the dexamethasone dependent decrease of the C3-protein reflects different abundancies of the C3-mRNA in hormone treated and contro

ABLE 2. RESPONSE OF THE C3-PROTEIN BIOSYNTHESIS TO DEXAMETHASONE

	pro-C3		C3α						
examethasone oncentration M	10^{-6}	0	0	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	$10^{-6}/0$
pm/band 10^{-3} a)	5.8	11.8	12.0	13.9	12.2	14.8	5.8	4.8	7.4
of control	49	100	100	116	102	123	48	40	62

) Pro-C3 and C3α bands were excised from the gel in fig. 2, solubilized and ounted. The values are corrected for differences in the incorporation of ethionine into total newly synthesized proteins.

ells, the C3-mRNA concentrations in these cells were compared.

Total mRNA (9 µg), prepared from J774.2 cells maintained for 48 hrs in 0^{-6} M dexamethasone (fig. 3A, tracks 2 and 4) and from control cells (fig. 3A, racks 1 and 3) was size fractionated by electrophoresis in agarose gels. As hown by staining the RNA with ethidium bromide (fig. 3A, tracks 1 and 2) the verall mRNA patterns from hormone treated and control cells were not signifi- antly different. The relative amounts of C3-mRNA were determined by transfer of he RNA from the gel onto a nitrocellulose filter and subsequent hybridization o an excess (300 ng) of a radioactive cloned cDNA probe, specific for C3-mRNA 9). The autoradiograph in fig. 3A shows that the C3-mRNA is more abundant in he sample from hormone treated cells (track 3) than from control cells (track 3). he amount of hybridized cDNA in both bands was determined by scintillation ounting and showed a difference of 1.7-fold. The reliability of this measure- ent is demonstrated in fig. 3A, tracks 5-7, where decreasing amounts of total RNA from mouse liver were loaded in each track. The amount of hybridized C3-cDNA as precisely proportional to the amount of liver mRNA (fig. 3B). In three inde- endent mRNA preparations, the abundancies of the C3-mRNA were determined to be .5 ± 0.2 fold higher for dexamethasone treated cells than for control cells. hus there is an opposite effect of the hormone on the steady state level of the 3-mRNA and on C3-protein synthesis.

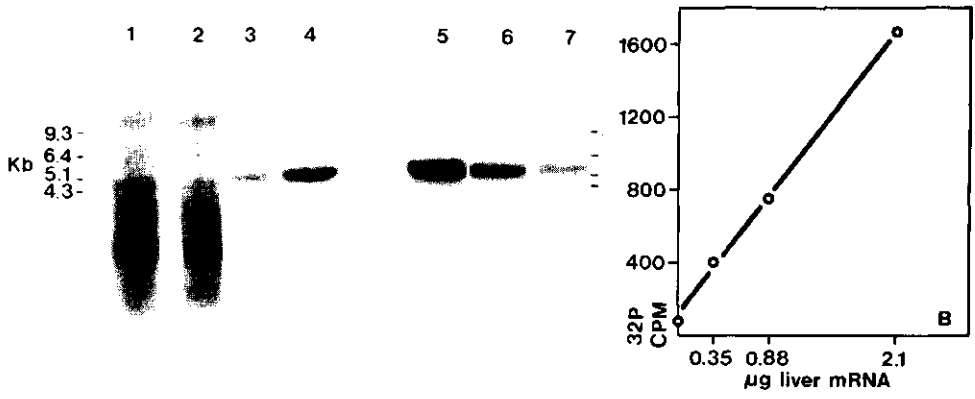


Figure 3. Titration of C3-mRNA

A. mRNA samples were denatured in glyoxal and fractionated by electrophoresis through an 0.7% agarose gel. The RNA was stained with ethidium bromide, tracks 1 and 2, or transferred onto nitrocellulose and hybridized to a radioactive clonec cDNA probe for C3-mRNA (9), tracks 3-7. Tracks 1 and 3: 9 µg of mRNA from J774.2 cells. Tracks 2 and 4: 9 µg of mRNA from J774.2 cells treated for 48 hrs with 10^{-6} M dexamethasone. Tracks 5-7 decreasing amounts of mouse liver mRNA: 2.1 µg, 0.88 µg and 0.35 µg respectively. E.coli tRNA (1 µg) was added as carrier to samples 6 and 7. B. Determination of the amount of hybridized cDNA in tracks 5-7 C3-bands were excised and counted in scintillation liquid.

Discussion

Metabolic labeling of proteins for a short time followed by immunoprecipitation and determination of the amount of radioactivity in specific protein bands, provides a reliable method to quantify relative rates of biosynthesis of specific proteins. Because the poolsize and specific activity of the radioactive precursc might vary inside the cells in different cell cultures, the results have to be normalized with respect to the total amount of radioactivity incorporated in newly synthesized protein.

Macrophages. Resident mouse peritoneal macrophages were compared with starc induced peritoneal macrophages for the rates of biosynthesis of C3 and C4-protei The resident cells synthesized substantially (5 and 20-fold) more C3 and C4 than induced cells. For C3 no similar observations have been published but for the guinea pig C4 opposite results have been reported (11). In that report the biosynthesis of C4-hemolytic activity was shown to increase upon starch induction, while the fraction of C4 producing cells remained constant and no significant change in the C4-protein (radioactive antigen) synthesis was detected. It is dif

icult to compare the results on the radioactive labeling in this report (11) ith ours, because the authors normalised their data with respect to the number f cells or the amount of radioactivity incorporated in secreted proteins. In our ands the secreted proteins represented only 5% of the newly synthesized protein data not shown) and therefore we normalized with respect to the radioactivity ncorporated into the proteins in the cytoplasmic extract. An inherent difference etween mice and guinea pigs could also explain these divergent results.

Cell lines. We demonstrated the biosynthesis of C3-protein in tissue culture ell lines of both hepatic and macrophage origin. Previously it has been repor- ed (12) that several rat hepatoma lines produced and secreted C3-activity. We ave tested the subline FaO (8) of the cell line H4 (12) and confirmed the ear- ier observation on the protein level by metabolic labeling and immunoprecipita- ion. In addition we found that also the HTC line (7) synthesized C3-protein al- hough at a much lower level. For macrophage derived cell lines no clearcut evi- lence of C3 synthesis has been reported. We found the IC21 (5) and J774.2 (6) ines to be strong producers of C3-protein. Although the IC21 cells synthesized ore C3-protein, the J774.2 cells were chosen for further investigations because f their shorter generation time.

The IC21 and J774.2 lines were also tested for the biosynthesis of C4-pro- ein. The J774.2 line did not produce detectable amounts of C4 and with the IC21 ine we obtained only weak indication for the synthesis of some C4 related mate- ial (data not shown). It is interesting that both these cell lines produce much ore C3-protein than C4-protein whereas for primary macrophages we found about a ive fold higher synthesis of C4 than of C3, especially because the origin of hese cell lines is very different. The IC21 line was obtained by transformation f macrophages with SV40 virus and the J774.2 line generated from a natural tumor f the reticulo-endothelial sytem (reticulo-sarcoma).

Some of the experiments with cell lines and primary macrophages have also een performed using a direct immunoprecipitation method (9). Because cold com- lement proteins from plasma were co-precipitated with the radioactively labeled roteins in those experiments, it was possible to compare directly the sizes of the subunits of the components in plasma with the ones syntheszed in tissue cul- ture. Whereas for C3 no differences were observed, the C4α chain from macrophage C4, synthesized in vitro, was measured to be approximately 5000 daltons larger than the C4α chain in plasma. Similar observations have been reported earlier (25).

Effect of dexamethasone. Dexamethasone, a synthetic glucocorticoid hormone, is a potent anti-inflammatory agent. For both liver cells and macrophages it has been reported that the secretion of several proteins is affected by glucocorticoids (13-16,26), eg. elastase, collagenase and plasminogen activator. C3 synthesis as measured by by hemolytic activity, has been reported to increase up to nine-fold upon addition of hydrocortisone to the medium of the rat hepatoma cell line H4 (12). We tested a subline (FaO) of the H4 line for its response to dexamethasone, but no significant effect was observed. Possibilities to explain this difference are a) the subline has lost its glucocorticoid response, b) a different glucocorticoid hormone was used, c) hemolytically active C3 is not proportional to radioactive antigen. The rat hepatoma line HTC however, showed a two-fold decrease in the C3-protein synthesis. Because in the same experiments we observe a two-fold increase in the synthesis of fibronectin upon addition of dexamethasone, the decrease in C3-protein synthesis is not simply due to a toxic effect of dexamethasone. Also for primary hepatocytes it has been shown that the amount of fibronectin can be increased by incubation in presence of dexamethasone (27). Fibronectin was identified by its molecular weight, affinity for Staphylococcus aureus and gelatin-sepharose (16) and in independent experiments by immunoprecipitation (14).

The macrophage line J774.2 also responded to dexamethasone with a two-fold decrease of C3-protein synthesis. Because these cells produce much more C3 than the HTC cells, further characterisation of the dexamethasone response was performed on J774.2 cells. The decrease in the C3-protein synthesis occured between 10^{-8} and 10^{-7} M dexamethasone. This is very similar to results obtained for the secretion by macrophages of proteases such as collagenase and elastase (14). The concentration of glucocorticoids in our fetal bovine serum was measured to be below 10^{-11} M (data not shown) and therefore their contribution can be neglected

In most of the studied cases where steroid hormones influence the expressio of genes (i.e. egg-white and egg-yolk proteins with estrogens), the change in the steady state concentrations of the mRNAs can account for the effect observed on the protein synthesis. We have tested wether this is also true for the C3-mRNA concentrations in J774.2 cells in presence and absence of dexamethasone. Although reproducibly a two-fold decrease was found for the C3-protein synthesis the mRNA concentration for C3 was reproducibly 1.5-fold higher in the presence of dexamethasone. This means that dexamethasone must have multiple effects on these cells, affecting in opposite ways the transcription and translation. Stu-

lies on the expression of proteins and mRNA of mouse mammary tumor virus (MMTV) genes in MMTV infected HTC cells (28) and in L cells transfected with MMTV-DNA (18) demonstrated a large increase in the concentration of virus specific RNA upon the addition of dexamethasone to the culture medium, but only a several fold lower increase in the synthesis of viral proteins. The lower efficiency of translation of specific mRNAs could be due to an influence of the hormone on the translational apparatus or to an accumulation of aberrant (untranslatable) mRNAs. These possibilities could be tested by in vitro translation studies.

The size determination of C3-mRNA in fig. 3A, using glyoxylated mRNA, differs substantially from the value we reported previously. There methyl-mercury-hydroxide was used for the denaturation of the mRNA and a value of 7500 nucleotides was determined (9) in stead of 5300. Further experiments have to be performed in order to establish what the correct value is.

The method used for the comparison of C3-mRNA concentrations (fig. 3), provides an additional tool for the detection of the expression of the C3-gene and for comparative studies on the effects of regulatory stimuli, such as anti-inflammatory agents, on the expression of the C3-gene.

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5 CONCLUDING REMARKS

The mayor part of the work reported in this thesis, aimed at the generation and isolation of DNA probes complementary to the messenger RNA for the complement components C3 and C4. Indeed for the complement component C3 the isolation of a complementary DNA probe has been successful and therefore it has become possible to perform detailed studies on the gene(s) and the RNA transcripts for this component.

Recent results (unpublished[1]) revealed that among the clones harboring recombinant plasmids (310, see chapter 3) eleven (3%) contained C3-specific sequences. Nine of these contained different plasmids with partially overlapping C3-cDNA inserts, covering in total 4700 nucleotides (90%) of the C3-mRNA.

The C3-cDNA probes are now used for a variety of experiments in different lines of investigation. a) Further characterization of C3-mRNA. In chapter 3 the C3-mRNA was estimated to contain 7500 nucleotides. This value was obtained by electrophoresis of mRNA in presence of the denaturing agent methyl mercury hydroxide. In chapter 4, using glyoxal for the denaturation of the RNA, C3-mRNA was found to contain only 5300 nucleotides. several independent experiments i.e. primer extended reverse transcription and digestion of C3-mRNA with RNase H after hybridization to C3-cDNA fragments, indicate that the value of 5300 probably is correct. Also the length of the mRNA for vitellogenin, which has a similar size as C3-mRNA, has been overestimated, using the methyl mercury hydroxide method (Dr. G. Ryffel, personal communication). b) Gene expression. Several aspects of the regulation of the expression of the gene for the complement component C3 have been studied in chapter 4. Sofar no convenient model system for the modulation of the expression of the C3-gene has been found, in which it will be possible to study the mechanism of control of gene expression. The continuous presence of complement component C3 might be so essential that its gene is expressed constitutively in the producer tissues and that major regulation never takes place. In any case, if in future regulatory stimuli will be found which have a strong effect on the expression of the C3-gene, tools for a detailed study of the mechanism are available. c) Gene structure. The mouse and human C3-genes have been analysed by digestion with several restriction enzymes, followed by size fracti-

1) All the unpublished results which are mentioned in this chapter were obtained in a collaboration of the following persons (in alphabetical order) : Horst Domdey, George Fey, Verena Müller, Karel Odink and Karin Wiebauer.

onation and detection of the C3-specific fragments by hybridization to radioactively labeled C3-cDNA. Fragments of the C3-gene, comprising at least 90% of the sequences coding for C3-mRNA, have been isolated from a mouse gene library. Results from several experiments are to be expected soon : the determination of the number of C3-genes in the mouse and human genome; the location of the human gene(s) on a chromosome(s), with the use of human-mouse hybrids cells; sequence analysis of parts of the C3-cDNA, which will possibly reveal the amino acid sequence of the C3a polypeptide and of the portion of C3b which contains the bindi site for cells and particles (see 1.3.4 and 1.3.5).

All these examples show that a new approach has become available to answer questions about the expression, genetic aspects and structural aspects of the gene for the complement component C3.

For the complement component C4 the isolation of a complementary DNA probe has not been successful so far. The serum level of C4-protein is approximately three fold lower than for C3-protein. This might reflect a lower abundancy of th C4-mRNA in the liver. Indeed, after in vitro translation of liver mRNA and immun precipitations using anti-C3 and anti-C4 sera, much less (at least ten fold) pro-C4 was found than pro-C3 (chapter 3). But low efficiency of the immunoprecipitation with anti-C4 could also explain these results. However, if macrophage mRNA is used for in vitro translation, the immunoprecipitations with anti-C4 and anti-C3 are equally efficient. Therefore it is likely that part of the polypeptides, obtained after in vitro translation of liver mRNA, which have a molecular weight of 190'000 is not related to pro-C4 and that indeed C4-mRNA appears approximatel ten fold less abundant than C3-mRNA in liver. This in turn could explain why no recombinant plasmids were found which contained cDNA inserts for C4-mRNA. A larger collection of clones containing recombinant plasmids should be made in order to increase the chance of isolating a C4-cDNA probe. A C4-DNA probe would provide an entrance into the major histocompatibility complex and could help to elucidate whether there is a relation between this complex and the complement system, other than the location of some of the complement genes within the major histocompatibility complex.

It is conceivable that in the future, apart from the complement components C3 (and C4?), for the other components of the complement system cDNA clones will b isolated. However, serious problems might be encountered due to low abundancies of their mRNA. If these isolations will be succesfull, the coherance in the regulation of the biosynthesis of the complement components might become understood.

6 SAMENVATTING

Het complement systeem vormt een belangrijk verdedigingsmechanisme tegen infecties in de mens en andere vertebraten. Bij de activering van dit systeem, die door antigeen-antilichaam-complexen en verder door een groot aantal andere substanties geïnduceerd kan worden, zijn ten minste zestien serumeiwitten betrokken. De activering van het complement systeem verloopt via een waterval-achtige reeks van eiwitsplitsingen en de vorming van eiwitcomplexen. Het uiteindelijke resultaat is dat de infectieuze substantie snel uit het organisme wordt verwijderd.

Het is onduidelijk hoe de contrôle van de expressie van de genen van het complement systeem verloopt en hoe wordt geregeld dat de juiste hoeveelheden verschillende complement eiwitten worden gesynthetiseerd. Een onderzoek naar de structuur van de betrokken genen en een analyse van de overschrijving van deze genen in boodschapper-RNA kan tot de verheldering van deze problemen bijdragen. Verder kunnen deze studies bijdragen tot het begrip van genetisch bepaalde gebreken in componenten van het complement systeem.

De ontwikkeling van de zogenaamde recombinant DNA technieken heeft het onlangs mogelijk gemaakt deze problemen aan te pakken. Boodschapper-RNA kan als matrijs worden gebruikt voor de kunstmatige synthese van complementair-DNA (cDNA). Dubbelstrengs cDNA kan in het DNA van een bacterieel plasmide worden ingevoegd. Het op die wijze gemaakte "recombinant" DNA kan worden gebruikt om bacteriën te transformeren, waarna grote hoeveelheden schoon cDNA uit rein-culturen (cloons) van deze bacteriën kunnen worden geïsoleerd. Aangezien dit DNA complementair is aan een boodschapper-RNA en aan het bijbehorende gen, kan het worden gebruikt om dit RNA en het gen te herkennen en wordt het daarom "DNA-probe" genoemd.

Het voornaamste doel van het hier beschreven onderzoek was een DNA-probe te isoleren voor de derde en vierde component van het complement systeem.

In hoofdstuk 1 wordt beschreven welke strategie is gevolgd om deze DNA-probes te isoleren. Verder wordt het mechanisme van het complement systeem in het kort uiteen gezet en wordt de huidige kennis van de biosynthese van de componenten samengevat.

Hoofdstuk 2 handelt over de gedeeltelijke zuivering van component C4 uit muizeplasma en over de bereiding van een monospecifiek antiserum tegen muize-C4. Antisera zijn van essentieel belang voor de identificatie van eiwitten en daardoor voor de identificatie van boodschapper-RNA door vertaling in een cel-vrij eiwitsynthetiserend systeem. Voor C3 zijn antisera in de handel verkrijgbaar.

In hoofdstuk 3 wordt beschreven hoe de C3- en C4-boodschapper-RNA's door

vertaling in vitro zijn gekarakteriseerd en hoe deze RNA's gedeeltelijk zijn gezuiverd. Verder wordt beschreven hoe een RNA-preparaat, dat verrijkt was in C3- en C4-RNA, is gebruikt in de bovengenoemde cloningsprocedure en hoe drie bacterie-cloons die C3-cDNA bevatten zijn geïsoleerd.

Hoofdstuk 4 handelt over de bestudering van enige aspecten van de biosynthes van C3-eiwit door primaire muizemacrophagen en door diverse typen cellen in weef selkweek. De C3-cDNA-probe is daarbij gebruikt om C3-boodschapper-RNA concentraties te vergelijken met de mate van C3-eiwitsynthese. Het effekt van het hormoon dexamethason, dat als krachtig anti-ontstekingsmiddel toepassing vindt, is bestudeerd. Merkwaardig genoeg is daarbij gebleken dat dit hormoon een tegengestel effekt heeft op de concentratie van het C3-boodschapper-RNA en de C3-eiwitsynthese in cellen in weefselkweek. De eerste bleek toe te nemen terwijl de tweede afnam.

In hoofdstuk 5 wordt een nabeschouwing gegeven over wat mogelijk is geworde nu C3-cDNA beschikbaar is. Verder wordt ingegaan op de vraag waarom het nog niet is gelukt een C4-cDNA te isoleren en hoe wellicht in de toekomst meer succes kan worden geboekt.

CURRICULUM VITAE

Karel Odink werd op 28 februari 1953 geboren te Naarden. In 1971 behaalde hij het diploma gymnasium β aan het Gemeentelijk Gymnasium te Hilversum, waarna hij zijn studie aan de Landbouwhogeschool te Wageningen begon. In 1978 behaalde hij het doctoraal examen in de Moleculaire Wetenschappen, met als verzwaard hoofdvak Moleculaire Biologie en als tweede hoofdvak Virologie. Zijn praktijktijd evenals zijn promotie onderzoek verrichtte hij aan het Zwitserse Instituut voor Experimenteel Kanker Onderzoek (ISREC) te Epalinges (Lausanne), aan de afdeling moleculaire biologie. Per 1 juli 1981 treedt hij in dienst bij de Wellcome Research laboratories, Beckenham, Engeland.